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Accelerated Biodegradation of Cellulosic Substrates.

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ACCELERATED BIODEGRADATION OF CELLULOSIC SUBSTRATES

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ABSTRACT

Utilization of appropriate chemical/physical/microbiological techniques for pretreatment of cellulosic substrates was shown to significantly accelerate cellulose biodegradation processes under both laboratory and in situ marine conditions. Initially an overlay plating system was developed for the isolation of cellulolytic bacteria. The procedure involved a double-layer agar system, with the basal portion consisting of a mineral salt/yeast extract agar and the upper portion, an alkali-treated cellulose-agar overlay. The system was shown to have limited application in the enumeration of cellulolytic bacterial populations.

An in situ rate of 3.8 mg solubilized/24 hr/gram substrate was determined for the decomposition of untreated, purified cellulose. Introduced (purified cellulose) substrate was degraded, in situ, at a rate 2.7 times slower than that for the indigenous substrate, Spartina. This indicates that processed cellulose wastes are even more recalcitrant than are natural cellulosics. A combination of nitrite-photochemical, alkali, and bacteria seeding pretreatments was shown to increase the rate of cellulose solubilization by a factor of 6.5.

In laboratory studies, pretreated cellulose samples

were solubilized in cadoxen and analyzed for determination of their degree of polymerization (DP). Ball-milling or UV-nitrite irradiation lowered the DP to a greater extent than did alkali treatment or UV-no nitrite irradiation. However, bacterial degradation rates for the cellulosics indicated that in effecting an increase in cellulolytic rates, DP is of secondary importance compared to the degree of swelling or bacterial accessibility of the cellulose fiber. Low DP, highly crystalline substrates were shown to have lower digestion rates than did higher DP, less crystalline celluloses.

Pretreatments for accelerating cellulose biodegradation were also examined with respect to their action on compounds occurring concomitantly with cellulosics. Electron-capture gas chromatographic studies indicated that a 90+% reduction of polychlorinated biphenyl (PCB) residues in cellulosics could be achieved by appropriate UV irradiation. PCB's in untreated cellulosic substrates were shown to remain stable throughout conventional cellulose decomposition processes and to be concentrated in the microbial-cellulose biomass during biodegradation. A proposal was presented which suggested incorporation of UV irradiation treatment into waste cellulose-SCP systems (or any recycling processes) where bioconcentration of PCB residues may present a significant potential danger.

The study indicated that environmental considerations of accelerated cellulose biodegradation parallel similar

problems in microbial SCP-cellulose processes. Objectives of such increased cellulose utilization technology include maximizing substrate conversion to desirable compounds concurrent with removal of toxic materials. Substrate pretreatment techniques were shown to have potential as solutions in meeting the above objectives.

INTRODUCTION

Current awareness and research are being focused with increasing emphasis on the problem of solid waste disposal. More than 250 million tons of urban waste, comprised of paper products, newsprint, cardboard boxes, rags, etc., is produced in the United States alone. Of this total amount, cellulose constitutes about 40-50% (Vaughn, 1970). The Council on Environmental Quality (1970) stated that quantities as great as 50 million tons of solid waste per year are being dumped at sea by the United States. These vast influxes of man-made cellulosic wastes, coupled with natural cellulose depositions, place a tremendous stress on environmental systems for necessary degradation and recycling of these materials.

Bohn (1972) suggested that this stress or pollution is actually a problem of "misplaced" waste. Natural mechanisms obviously incorporate great portions of cellulosic wastes into various food cycles. Yet, numerous studies, such as those by Jannasch et al. (1971) indicate that degradative mechanisms may be quite slow in some environments, especially in the marine environment beyond the neritic zone. In a suitable environment, cellulose assimilation into the biosphere may be quite rapid;

however, if "misplaced" or improperly placed, i.e., in deep sea, cellulose may persist as a recalcitrant pollutant. It is vital, then, to examine the possibilities of preconditioning cellulosic substrates to hasten their recycling into normal food chains. Ancillary to the thrust toward accelerated cellulose biodegradation, is the concept of increased utilization of cellulosic substrates in alternate processes. An example of this is seen in the microbial production from cellulosics of single cell protein or its conversion into commercially valuable products, such as glucose or ethyl alcohol. Developments in accelerated biodegradation as well as increased utilization of cellulose both serve as means to reduce the stressful impact of excessive cellulosic wastes on the environment.

The development of economically feasible technology is needed to cope with the aforementioned accumulation of cellulose which occurs annually in agricultural and municipal waste streams and tax already replete natural processes for cellulose recycling. As recently as June, 1974, the U.S. Environmental Protection Agency has stated that the development of such processes as microbial protein production from cellulosic wastes must be viewed as "essential, immediate, and futuristic technology" (Rogers, 1974).

Organic material exists in the marine environment as dissolved material and in the form of particulate matter or detritus which may range from micron to significantly

larger size. While the particulate portion represents only a fraction of the total organic composite, it is vitally important as a repository of recalcitrant energy sources. Parsons (1963) devised a relative scale from 0-100, arbitrarily approximating the distribution of total organic matter in seawater as follows: soluble organic matter, 100; particulate detritus, including microorganisms, 10; phytoplankton, 2; zooplankton, 0.2; and fish, 0.002. Chemical analyses by Parsons indicated that the detritus is 30% carbohydrate, 70% of which is crude fiber. Conservatively estimating 35 to 40% of the fiber as cellulose, one can readily appreciate the considerable portion of marine organic material existing as polymeric carbohydrate.

Cellulose detritus is even more abundant in the salt marsh and estuarine areas of Louisiana where production by the predominant plant, Spartina alterniflora, reaches 3 kg dry wt/m^2/yr (Alexander, 1972). Spartina detritus serves as the major storage, transport, and buffer mechanism in estuarine ecosystems as follows:

1) Organic material produced during the growing season may be stored and released later.

2) Detritus may be transported considerable distances away from the source of primary production.

3) Detritus in food chains may serve as a reserve food source during seasons of low primary productivity.

Thus, since cellulose is a basic component of organic detritus, it is essential to understand both the mechanism

and rate of its biodegradation.

Knowledge of cellulose degradation rates may provide an early warning method of monitoring marsh productivity. The subtle influences of extant stress factors, i.e., low level thermal, hydrocarbon pollution, etc., on this fundamental step in overall productivity may have far-reaching effects at the apex of the marsh food web.

A similar situation, man-made cellulosic inputs vs natural production, exists with respect to the fate and effect of disposed paper products in the marine environment. Manheim et al. (1970) found processed cellulose fibers to be especially abundant in surface waters near port areas, shipping traffic lanes, and untreated municipal sewage discharge areas. The cellulose fibers collected in the open ocean appeared totally undegraded while those recovered in the New York Bight area appeared pitted, eroded, and associated with a microbial mass. It was speculated that biodegradation of the fibers was more rapid in nearshore waters than in the open ocean. However, few definitive experimental data are available regarding fate and effects of the disposal of cellulosic materials in the marine environment.

Considerable information exists on the cellulolytic activities of terrestrial microorganisms. Also, considerable attention has been given to the area of accelerated decomposition of various cellulosic wastes under controlled

conditions, as a source of single cell protein to help supplant world protein shortages. In contrast, data on cellulosic biodegradation processes in the natural marine habitat are quite limited. Therefore, objectives of this study include:

1) Evaluation of rates of decomposition of cellulosic wastes in the marine environment.

2) Development of an improved method for the isolation of cellulolytic microorganisms.

3) Examination of methods for increasing rates of cellulosic waste decomposition in the marine environment.

4) Study of the fate of compounds insidiously associated with cellulosic wastes, e.g., polychlorinated biphenyls.

REVIEW OF LITERATURE

General Characteristics of Cellulose

Cellulose is by far the most abundant of macromolecules. On a global scale, quantities on the order of 1000×10^8 tons/year are synthesized and degraded, compared to 1000×10^6 tons/year for chitin (Hess, 1928; Tracy, 1957). Because of the large amounts produced each year, coupled with its relative resistance to rapid biological degradation, cellulose provides a reserve source of energy in nature. This energy pool acts as a stabilizer which tends to smooth out wide fluctuations in supply of energy to many parts of the biosphere (Colvin, 1972).

Cellulose is a generic name for a high polymer of glucose (poly 1, 4-β glucopyranose) in which the number of monomer units in the chain is usually between 100 and 3000 (Fig. 1). The number of linked units is termed the degree of polymerization (DP). Degradation of cellulose usually implies the lowering of DP by chain scission and may take place, according to Ott (1963), either with or without chemical changes to the glucose units remaining in the chain fragments.

Properties of cellulose depend upon its DP and upon the arrangement of its chains in the fiber complex. There

is general agreement that in nature the long, polyglucosan chains are associated to form an insoluble entity called a microfibril which may vary from 10 to 25 nm in width. The fibrillar nature of cellulose was recognized almost as soon as the electron microscope became available. Preston (1948) made early observations on cellulose microfibrils in the alga Valenia. Although most workers agree that the microfibrils may be arranged in a specific crystalline lattice, Colvin (1972) notes that the supramolecular manner of association of the chains within the microfibril is a point of controversy. Various association models are dealt with in a study by Manley (1971).

The paper industry has maintained a rather arbitrary characterization of various DP celluloses, in order of decreasing crystallinity (Escobar, 1971):

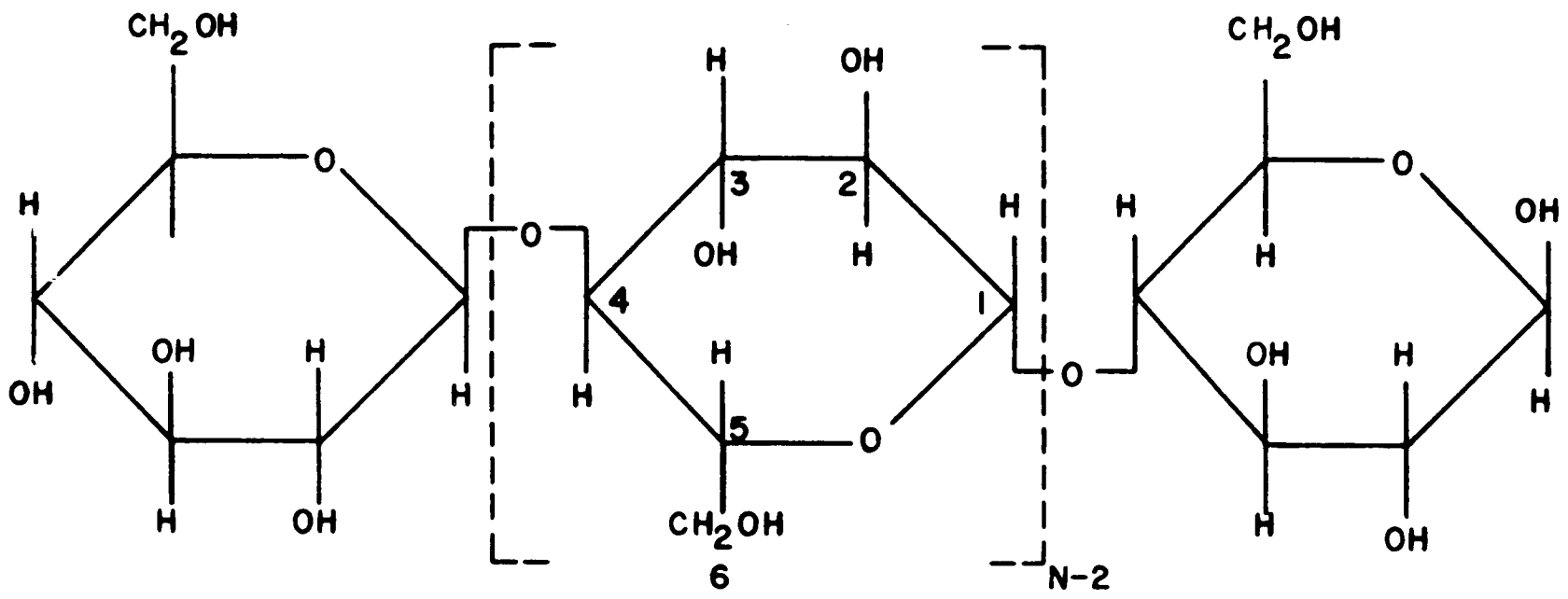
1) Alpha-cellulose--the more stable portion of cellulose is defined on the basis of its insolubility in 17.5% sodium hydroxide solution at 20 C. DP is greater than 200.

2) Beta-cellulose is that fraction solubilized by the above alkali solution but capable of being precipitated by neutralization at 20 C. DP of beta cellulose ranges from about 30 to 200.

3) Gamma-cellulose is the fraction remaining in solution after neutralization of the above alkali. DP is less than 30.

Native cellulose also contains varying amounts of non-cellulosic components. These include the hemicelluloses which are by definition those constituents of the plant cell wall which may be extracted by dilute alkalies.

Figure 1. Haworth chemical structure of cellulose. (from Tyagi, 1972)



Haworth Chemical Structure of Cellulose

Xylan is a hemicellulose commonly associated with most structural cellulose (Ott, 1963). Lignin is a complex, aromatic polymer which together with cellulose and hemicellulose, forms what is known as a lignocellulose complex. The relative amounts of lignin present in such a complex greatly influence the ease of its degradation (Norkrans, 1963). Cowling (1958) noted that lignin influences fungal degradation by physically blocking both extracellular enzymes and mycelia from cellulose, and by increasing the complexity of the enzyme system necessary for solubilization of the lignocellulose matrix because of the chemical and physical complexity of the tissue itself.

Measurement of Cellulolytic Activity

Cellulase System

The terms cellulase activity, cellulolytic activity, and cellulose degradation are used interchangeably in cellulose science. However, it must be noted that the use of these terms refers to a cellulase system in general and not necessarily to a single, specific activity. Recent reviews of the cellulolytic enzyme systems have been prepared by Hajny and Reese (1969), and Whitaker (1971).

A multi-enzyme hypothesis for enzymatic hydrolysis of cellulose was introduced by Reese et al. (1950). The sequence was schematically presented (Reese, 1956) as:

$$\text{Native Cellulose} \xrightarrow{C_1} \text{Linear Cellulose Chain} \xrightarrow{C_x} \text{Cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{Glucose.}$$

Both the C_1 and C_x components are necessary for hydrolysis of native cellulose. Microbes with just the C_x portion can hydrolyze cellulose only after some modification of the substrate.

Currently, the cellulase system is still thought to be composed of several components which act in sequence on the cellulose polymer (King and Vessal, 1969). The least understood component of the complex is probably C_1, which is thought to act primarily on highly oriented, crystalline cellulose. C_1 acts synergistically with two C_x components:

Cellulase component Cx_1: Exo-β-1-4 glucanase which successively removes single glucose units from the non-reducing end of the cellulose chain.

Cellulase component Cx_2: Endo-β-1-4 glucanase which cleaves the cellulose molecules randomly, the terminal linkages generally being less susceptible to attack than internal linkages.

Many methods of evaluating cellulose degradative activity have been developed. Eriksson (1969) discussed seven of the most commonly used assay methods: 1) Gravimetric determination of insoluble substrates, 2) Changes in mechanical properties of films or fibers, 3) Decrease in viscosity of solutions of cellulose derivatives,

4) Measurements of clearing zones in cellulose agar, 5) Determination of reducing end groups, 6) Turbidity of cellulose suspensions, and 7) Colorimetric determination of released soluble portions of cellulose derivatives.

A radiochemical method of assaying cellulolytic activity was reported by Koleff et al. (1972). The assay used ^{14}C-labeled cellulose as a substrate.

King (1964) noted that the ease of using soluble cellulose derivatives (carboxymethyl cellulose, CMC) in "cellulase" assays has led many contemporary workers to use CMC-ase activity as an indication of the cellulolytic nature of an organism. Reese and Levinson (1952), however, showed that many organisms are capable of hydrolyzing CMC, but are not at all able to degrade insoluble cellulosic substrates. The use of CMC-ase activity in the enumeration of cellulolytic microorganisms may give misleadingly high population estimates. Such data, however, may be useful as an indication of the necessity for cellulolytic organisms to compete with non-cellulolytic organisms for soluble polymer fragments once the initial attack on cellulose has begun. King (1964) further suggests that perhaps CMC-ase (C_x type) hydrolysis acts to accelerate overall cellulose hydrolysis by continually removing products of C_1 action and converting them to lower oligosaccharides.

Biodegradation and the Ecology of Cellulolysis

Cellulose is constantly being degraded in the environment by a variety of natural mechanisms. Photolysis, microbial-enzymatic cleavage, and mechanical disaggregation all act to degrade the polymer chains.

The reports of Jones and Jannasch (1956), Riley (1963), and Seki and Kennedy (1969), have shown that most particulate matter in sea water is associated with an aggregate formation of bacteria and film matrix material which forms a semi-independent ecosystem from the surrounding sea water. Various mechanisms of bacterial attachment or film formation have been suggested (Floodgate, 1966), all of which indicate the importance of proximity of bacteria and substrate. The sequence of increased bacterial biomass, increased enzyme production, and thus greater substrate solubilization may be especially favorable for recalcitrant molecule degradation.

King (1964) observed three divisions in sequential microbial degradation of cellulose. Initially, an attachment or close physical proximity is followed by a disaggregation of cellulose fibers. Then, extracellular hydrolysis or pitting and eroding of the fibers occurs. Eventually, saccharified remnants of the cellulose chain are assimilated into the microbial biomass. The first of these processes, disaggregation, is thought to be a result of the C_1 component of the enzyme system, questionably described a

number of years ago by Siu (1952) as a "hydrogen bondase." Controversy still exists as to the enzymatic sequence of events that occurs as cellulose is degraded.

The occurrence of specific genera in marine film formation was examined by ZoBell (1943), Wood (1967), and Kadota (1956). The work of Kadota (1956) indicated that of 252 cellulolytic bacteria isolated from a marine environment, only three genera were represented, Pseudomonas, Vibrio, and Cytophaga. Alexander (1972) noted three additional genera, Erwinia, Flavobacterium, and Serratia, assocaited with marine materials and which exhibited cellulolytic activity. Studies by Hood (1970) indicated that the highest numbers of cellulose decomposers in a salt marsh were associated with the predominant flora, Spartina, and that lower cellulolytic populations were found in marshland soil and open water areas.

In situ Measurement of Cellulolytic Activity

Laurent (1969) observed that for many years investigators have tried to evaluate cellulolytic activity in various natural situations. He noted that enumeration techniques imply a close relationship between numbers of microorganisms and total degradative activity while, actually, this relationship may be quite remote. Similarly, Meyers (1968) found striking utilization of cellulose based on gravimetric determinations with accompanying

increase in fungal biomass; however, very little cellulolytic activity was noted in the culture broth. It follows, then, that certain measures of cellulolytic activity may give quite conflicting results as compared with other types of cellulose degradation measurements.

Most workers, however, have continued to use some of the rather indirect methods for evaluating intensity of cellulolytic activity. Meyers et al. (1960), Von Brandt (1956), and Knopp and Webber (1960) all used loss of tensile strength of twine or cellulose fiber as an index of degradative activity.

An assay utilizing submerged wood blocks was presented by Meyers (1968) and Meyers and Scott (1968). The method involved submerging two or more wood blocks which were held together "sandwich fashion." Inner unexposed and outer exposed surfaces could then be examined for qualitative estimates and quantitative comparisons of cellulose degradation.

A number of workers have used a "bag" method for evaluation of insoluble substrate degradation. Essentially, the substrate is placed in a non-biodegradable bag or wrapper and weighed before and after being exposed in the environment. There are several advantages and disadvantages to this procedure which will be mentioned in a later discussion.

A sea-grass, Thalassia testudinum, was used as the

assay substrate in a "bag" decomposition study by Zieman (1968). The investigation showed that grass samples decreased in dry weight by almost 60% in 2 months. Hood (1973) used the method to study chitin degradation. An average solubilization rate of 87 mg/day/gm-chitin was determined for a Louisiana salt marsh area.

Hofsten and Edberg (1972) measured rates of degradation of various cellulosic fibers enclosed in nylon bags. They found cellulose decomposition to be slow in unpolluted sea water, i.e., 10% loss of weight in 200 days, and relatively high in nutrient-rich estuarine areas, i.e., 30% loss of weight per month.

Acceleration of Cellulose Degradation

Processes designed to accelerate the biodegradation of cellulosic substrates have basic factors in common. If any one or combination of the following changes occur, then degradation may proceed more rapidly:

1) Disruption of lignin material releases more of the cellulose from the lignocellulose complex and lessens the occluding effects of lignin.

2) Lowering the relative degree of crystallinity, by processes such as swelling, makes the cellulose more easily penetrated by cellulase enzymes.

3) Decreasing the DP increases solubility and makes more enzyme susceptible, terminal ends available.

Studies by Norkrans (1956) and Walseth (1952) have shown that the resistance of celluloses to enzymatic breakdown is closely associated with their degree of

crystallinity.

Processes that have received the most attention in accelerated cellulose biodegradation are alkali treatment, grinding, and photodegradation. Other treatments, such as enzymic saccharification, are not included in this group because they were developed more as end processes rather than pretreatments for faster solubilization of cellulosics.

Surprisingly, hydrolysis by strong mineral acids or weak organic acids decreases the susceptibility of the residual cellulose to degradation. According to Cowling (1958), this occurs because more easily hydrolyzed amorphous material is removed and, possibly, some substituted derivatives of cellulose are formed.

Alkali Treatment

Alkali treatment acts to modify the substrate as mentioned above. A review of the effect of alkali swelling has been prepared by Warwicker et al. (1966). Swelling of cellulose by alkali is the method currently proposed for use in a project developed at Louisiana State University for production of single cell protein from cellulosic wastes. A schematic of the alkali treatment section of the LSU-SCP process is presented in Appendix E.

Mechanical Disruption

Grinding, ball milling, or mechanically degrading cellulose has also been shown to increase its susceptibility to biodegradation. A reduction in DP and increased ability to be used as a ruminant ration was noted by Tyagi (1972), for ball-milled wood chips. Similar experiments by Millett et al. (1970) showed that the digestion of wood chips by cattle could be increased by treatment with alkali or by grinding.

After mechanical disintegration, increased degradative rates are greater than can be accounted for by increased surface area alone. Apparently, cut surfaces are more susceptible to cellulolysis than native surfaces (Reese, 1956). Very extensive mechanical disintegration, with a ball mill, was reported to have resulted in depolymerization as well as increased surface area (Pew, 1957).

Photolytic Processes

The light sensitivity of cellulosic materials is well documented. An extensive review of the subject prior to 1968 was prepared by Desai (1968). In general, light causes a decrease in tensile strength and DP, and an increase in alkali solubility. Also, yellowing and browning occur with production of carbonyl and carboxyl groups along the cellulose chain (Desai and Shields, 1970). Conflicting results have been obtained in studies examining microbial deterioration of irradiated cellulosics. Wagner et al.

(1957), Kaplan et al. (1970), and Abrams (1951) found an increased resistance to microbial attack of cotton fiber after ultraviolet (U.V.) irradiation. Experiments by Reese (1957) indicated that cellulose irradiation by cathode rays (β-rays) increased resistance to fungal attack. The mechanisms responsible for increased resistance to biodegradation of irradiated cellulosics have been speculated upon by Kaplan et al. (1970). These workers have ascribed the resistance either to (1) selective deterioration of microbially susceptible amorphous cellulose, leaving more resistant crystalline cellulose; (2) removal of microbial nutrients such as trace salts or sugars, waxes, pectic substances; or (3) formation of substituted cellulose derivatives which may not be readily degraded by microbial action.

In contrast, the findings of Saeman (1952) and Lawton (1951) showed that there was no cross-linking of cellulose chains induced by irradiation and that depolymerization was random in both crystalline and amorphous regions. Supporting these findings is the work of Rogers et al. (1972), in which irradiated cellulose showed an increased susceptibility to degradation by fungi. This study utilized U.V. irradiation with low levels of nitrite present in a cellulose-water slurry. The action of nitrite in accelerating cellulose photolysis has been described by Schwartz and Rader (1967).

Ogiwara and Kubota (1973) speculate that there may be two kinds of photo-induced reaction--a direct reaction initiated by light and cellulose, and another initiation related to sensitizing actions depending upon the contaminants present in the cellulose. Eskins et al. (1973), working in the area of dye-sensitized photodegradation, studied a number of dyes which were capable of generating both free radicals and singlet oxygen and thus, possibly capable of acting as accelerators in cellulolysis. Results indicated that the dyes did increase photolysis, however, there was a reduced susceptibility of the photo-oxidized cellulose to enzyme degradation.

Work by Gilligan and Reese (1954) and King et al. (1964) offers some clarification to the above conflicting reports. It was found that irradiation initially increased resistance to microbial degradation. Disaggregated cellulose chains in the amorphous regions tended to recrystallize and made the substrate more resistant to enzymatic hydrolysis. Further irradiation then tended to reverse the recrystallization process and increase the susceptibility of the substrate to deterioration.

Desai and Shields (1969) showed that photodegradation of paper samples by U.V. irradiation was predominantly a surface effect. This study emphasized the need for slurrying and stirring of cellulosic substrates during irradiation in order to obtain uniform photolysis in spite

of low penetrability by U.V. irradiation.

Association of Polychlorinated Biphenyls with Cellulosics

Rationale for Examining PCB's in Cellulosics

Commercial papers and paper products are associated with a variety of toxic contaminants. Heavy metals, fire-proofing compounds, anti-fungal treatments, etc., all may occur within or as a coating on paper materials. Any utilization or disposal of cellulosics must involve consideration of the ultimate fate of these compounds. The necessity for examing the presence and levels of toxic compounds, such as PCB's, in paper products appears even more striking when one considers that man may be the final recipient of these compounds, e.g., as ingested food.

The current world protein shortage has necessitated the development of numerous novel and unconventional sources of protein. Researchers at Louisiana State University, Baton Rouge, have been active in developing processes for the production of single-cell protein from cellulosic wastes. Dunlap (1969) and Callihan (1971) have studied a sugar cane-bagasse degradation process ultimately for the production of SCP. Private industry is currently building up from pilot plant production to a commercial size facility to utilize the LSU process for production of microbial protein from cellulosic municipal wastes. The

desirability of these developments can be appreciated by examining the economics of the protein production (Appendices A, B). Some re-evaluation of processing methodology, however, may be warranted in view of real and potential toxic contaminants that are associated with paper substrates.

Another novel method of protein production that has been suggested is the feeding of paper products to agricultural livestock. Perhaps this practice also needs re-evaluation. Cattle will consume rations containing up to 20% paper and assimilate up to 90% of the dry matter of certain papers, such as brown cardboard (Mertens et al., 1971). Office paper has been successfully incorporated in sheep rations by Nishimuta et al. (1969).

The above developments for production of microbial or animal protein must be considered with respect to biological magnification. The latter is the ability of certain organisms to remove from the environment and store in their cell mass, substances normally present in the environment at nontoxic levels. The concentration of these substances becomes greater with each step in the food web.

It is known that compounds such as polychlorinated biphenyls (PCB's) have been used as dye carriers in specialty printing inks. High concentrations of PCB's have been reported as present in carbonless copying paper (Masuda et al., 1972). Also, there have been studies that

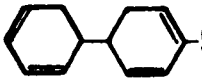
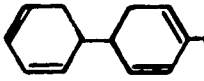
indicate that PCB's survive when specialty paper products are recycled for use in other paper products such as cardboard (Anonymous, 1972). Levi and Nowicki (1972) traced electron capturing peaks in extracts of cereal grains to the presence of corresponding PCB compounds in cloth bags.

Description of PCB's

PCB is a generic term that refers to a family of partially or wholly chlorinated isomers of biphenyl. A characterization of two commercial PCB compounds presented in Table 1 represents a typical formula and not an absolute description of a single compound. PCB's are colorless, highly viscous fluids which are quite valuable from an industrial standpoint. They are nonconductive, incredibly persistent compounds capable of withstanding temperatures of up to 1600 F. However, it is this persistence that makes them a hazard in the environment. Edwards (1971) further notes that the compounds are not hydrolyzed by water, acid, or alkali, and have a thermal stability enabling them to be used in fire-proofing. Interest in monitoring PCB's has increased because some of the physiological effects of the PCB isomers are similar to those of the chlorinated pesticides (Edwards, 1971). As methods for the detection of chlorinated hydrocarbon pesticides have improved, PCB's have been found in the environment by increasing numbers of investigators.

TABLE 1

Characterization of PCB Compounds

Compound	Average Composition	Average Molecular Weight	Molecular Structure
Aroclor 1254*	$C_{12}H_5Cl_5$	326	 54% Cl
Aroclor 1260	$C_{12}H_{3.7}Cl_{6.3}$	371	 60% Cl

*Numbering Protocol:

First two digits = Compound number
Second two digits = Percent chlorination

PCB's such as Aroclor, Phenochlor, and Clophen are incorporated as components in a variety of industrial products. Because of their low vapor pressures, they are used as vapor suppressants in lubricating fluids and insecticides to prolong the active life of an application. Veith and Lee (1970), as cited by Dube et al. (1972), reported finding PCB's in several detergents, aluminum foil, and packaging material. Senum et al. (1973) reported finding PCB residues in a survey study of over 100 papers and paper products. The paper sources studied included diverse samples from Playboy to paper bags and contained contaminating PCB residues of from 2 to 40 ppm.

Occurrence in the Environment

Reports on the widespread distribution of PCB's in the global ecosystem are increasing. Studies by Risebrough et al. (1968a, 1968b), Holden (1970), and Dube et al. (1974) have shown PCB contamination in many marine organisms such as mussels, oysters, algae, and sea birds. Dube et al. (1974) found levels of 42 ppb in the effluent of a wastewater treatment plant and concentrations 1000 times greater than this in the digester sludge of the plant. Oysters are considered to be ideal indicators of many types of pollution (virus, heavy metals, etc.) because of their filter-feeding mechanism. Subsequently, workers have reported concentrations as high as 70,000 ppm of PCB residues in oysters from heavily polluted areas (Ehrlich, 1970).

PCB's have been detected throughout the world in fish, birds, waterways, and in humans. In a study released by the U.S. Environmental Protection Agency (EPA), PCB's were found in samples of human fatty tissue with concentrations as high as 250 ppm (Pichirallo, 1971). Waddell (1974) indicates that at present, the Japanese government provides daily bulletins of inshore levels of heavy metal and PCB concentration. Also, it provides to the public maximum recommended human intake levels for different varieties of fish and mollusks taken from inshore areas.

Physiological Effects

The physiological-ecological effects of PCB's are still not fully understood. McLaughlin (1963) and Peakall (1967) found that PCB levels as low as 0.5 ppm in avian rations could cause altered hormone levels and reduced reproductive capacity. These workers also suggested that there are synergistic effects between some PCB's, DDT, and Dieldrin.

Acute effects of PCB's in humans have been described by Sax (1957) as including skin irritation, rash, and pigmentation of exposed areas. Internal damage to the liver may involve jaundice and liver atrophy. An incident involving leaked PCB's was reported in Japan, in 1968. Over 300 people developed skin irritations of varying severity, and babies showed symptoms of chlorobiphenyl jaundicing (Pichirallo, 1971).

Relatively low levels of PCB's may be disastrous in the marine food chain. A concentration factor of 100 in seven days was obtained in the algae, Chlorella (Sodergren, 1973). The implications of this type of concentration are frightening in light of studies such as those by Duke et al. (1970) in which tests on juvenile shrimp resulted in 100 percent mortality in 48 hours at a PCB level of 100 ppb. Although there is some information available concerning acute effects of PCB's in humans (Sax, 1957), there are few reports of chronic effects of low levels of PCB's.

As of February, 1972, the U.S. Food and Drug Administration (FDA) classified PCB's as a "potential, but not immediate health hazard." Pichirallo (1971) notes that the FDA guidelines place maximum levels at 5 ppm in fish and poultry, but recognize that re-evaluation is necessary. Currently, the FDA is reorganizing its policies concerning PCB contamination.

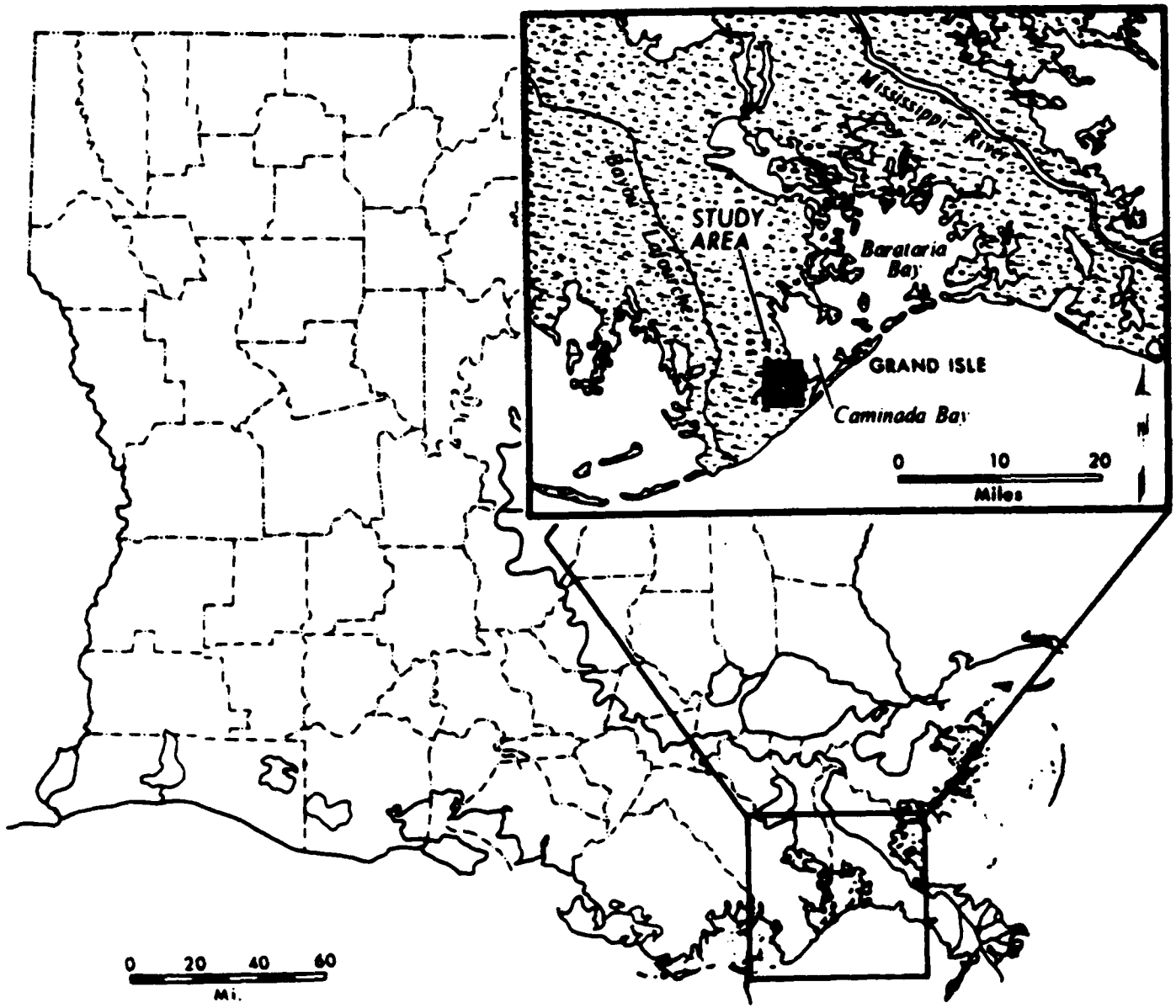
MATERIALS AND METHODS

This study involved a number of phases, all evaluating various aspects of cellulose degradation. Initially, samples of water, sediment, and detritus were taken from a salt marsh area and analyzed with several isolation techniques for cellulolytic bacteria. Rates of cellulose biodegradation for diverse substrates were then examined in a marsh lake and included pre-seeding experiments with the previously isolated cellulolytic bacteria. Subsequent cellulose studies were performed in the laboratory to aid in interpreting data from the environmental findings. In the concluding portion of the investigation, associations of polychlorinated biphenyl compounds with cellulosic biodegradation processes were examined using electron capture gas chromatography techniques.

Collection of Samples

Collection procedures have been described by several workers (Crow, 1974; Meyers et al., 1970) and will only briefly be discussed here. Collections were made from three areas in the Barataria Bay estuary (Figure 2). These sites were selected to augment as well as draw upon previously determined baseline data from earlier studies. One site was within a shallow semi-enclosed lake, Airplane

Figure 2. Sampling location in the Barataria Bay area of coastal Louisiana


Mississippi River
Bayou Lafourche
STUDY AREA
Barataria Bay
GRAND ISLE
Caminada Bay
0 10 20
Miles
N
0 20 40 60
Mi.

Lake (Fig. 3, area between sites 7 and 8). The other two areas were near crude oil study plots, both described by Crow (1974), one at the entrance to Airplane Lake (site 5) and the other on a small island located northwest of Grand Isle, Louisiana. The island, Martigan Point, was within an area that received crude oil from a pipeline break in Barataria Pass in October 1972.

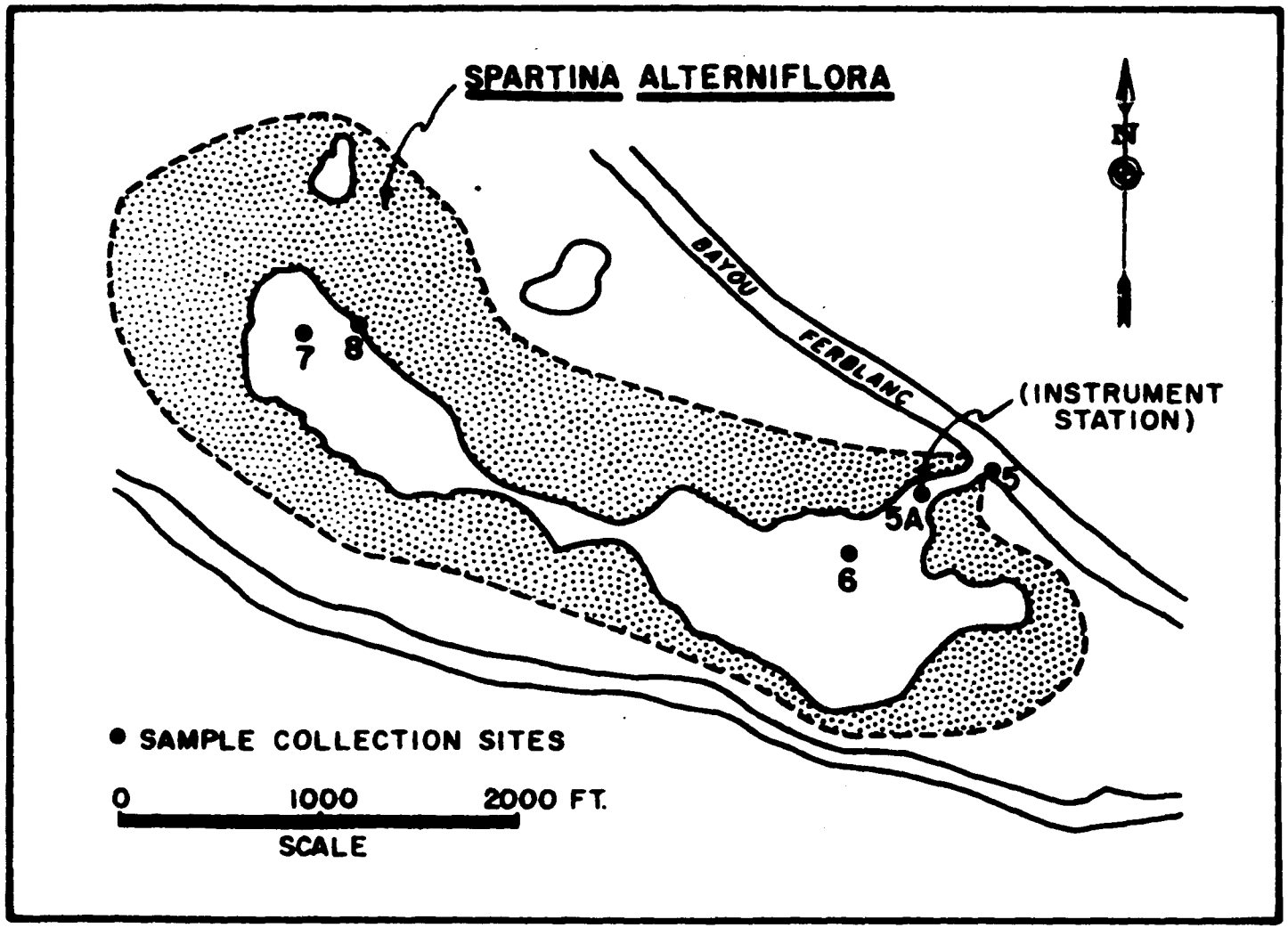
Sediment and Spartina detritus samples were collected at the sites in sterile petri plates. Water samples were taken in sterile 500 ml prescription bottles. The petri plates, in sterile Nasco-Whirl-pak bags, and water samples were then stored in an ice chest for transit to the laboratory. Analyses were initiated within 24 hours of sampling.

One deep water site was included in the study. Surface and meter-deep water samples were obtained in water approximately 100 meters deep, 30 miles south of Grand Isle, La. This pelagic sampling was taken to compare with samples taken from highly productive inshore areas.

Isolation Techniques

Three techniques, two of which involved enrichment procedures, were used for isolation of cellulolytic bacteria in this study. Initially, a procedure described by Rodina (1972) was attempted.

Figure 3. Airplane Lake. Sample areas 5,6,7,8.


SPARTINA ALTERNIFLORA
BAYOU FERBLANC
(INSTRUMENT STATION)
7
8
5
5A
6
N
● SAMPLE COLLECTION SITES
0 1000 2000 FT.
SCALE

A filter paper disk, Whatman #1, was folded to form a cone, which was then placed upright in 250 ml Erlenmeyer flasks containing 100 ml seawater with 0.1% Yeast Extract (Difco), and autoclaved. Aged natural seawater (ANSW) was used throughout for necessary dilutions in further manipulations of enrichment. The flasks were inoculated with appropriate dilutions of sediment, water, or detrital material and incubated for 3-7 days at room temperature (22 C) on a reciprocal shaker. As soon as visual degradation of filter paper, i.e. translucency or maceration, was observed, a loopful of the cellulosic pulp was removed and used to inoculate fresh flasks. This process was repeated several times to enrich for the aerobic and mesophilic cellulose-utilizing organisms. A portion of the pulp was then removed from the enriched culture, shaken with 10 ml of sterile ANSW, and streaked onto petri plates of the following media: Marine Agar 2216 (Difco), Basal Salts Agar (BSA) with 1% cellobiose, and BSA with 5% ball-milled, alkali-treated cellulose (see section on substrate preparation). The basal salts solution contained per liter of distilled water: NaCl, 15.0 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.1 g; and $CaCl^2$, 0.1 g. For solid media, 1.5% agar and 0.1% yeast extract were added. Carbon sources and other necessary ingredients were added as noted.

Subsequently, it was observed that satisfactory enrichment could be obtained using filter paper strips in test tubes instead of in flasks. Bellco-closure tubes were incubated at room temperature on a roller drum (Model TC-5, New Brunswick Scientific) at approximately 60 rpm to ensure aeration of the liquid.

Isolates were picked from the various solid media and re-tested for cellulolytic activity in the cellulose strip tubes. Cultures maintaining their degradative activity were restreaked onto BSA cellulose agar. Purity of the isolated organisms was confirmed by colony morphology and microscopic examination. Stock cultures were maintained on two media: Marine Agar 2216; Nutrient Agar (Difco), containing 1.5% NaCl, 0.1% yeast extract, and 5% alkali-treated cellulose and kept at 25 and 10 C. BSA and the stock nutrient agar are modifications of media used by Fleenor (1973) and Han (1969) adapted for marine bacteria. All media were adjusted to pH 6.8-7.0 after autoclaving, by aseptic addition of sterile 1% NaOH.

An additional isolation method included a modification of a technique suggested by Srinivasan (1974). This procedure did not necessarily require prior enrichment for cellulolytics. An agar overlay system was used in which the bottom layer consisted of approximately 10 ml BSA. This layer was then inoculated by either streak or smear procedures, incubated 6-10 hours at 30 C, and

microcolonies allowed to develop. Tempered overlay medium (45 C or below) containing cellulose was then added in a thin layer (about 5 ml). The overlay medium contained basal salts solution with 0.8% agar and 5% ball-milled alkali-cellulose. After 3-5 days at 30 C, cellulolytic activity was indicated by diagnostic cleared zones. Material from these zones was then picked and restreaked on BSA-cellulose agar for further isolation of cellulose degrading organisms.

Characterization of Isolates

All bacteria were characterized as to colonial morphology, pigment formation, gram reaction, and reaction on cellulose agar overlay medium. A selected number of isolates were tentatively identified as to genus according to the methods of Kadota (1956). The isolates which were chosen for further characterization, because of their vigorous cellulolytic activity, were used in subsequent rate studies of cellulose decomposition.

Substrate Preparations

The following were used as cellulosic substrates in this study:

1) Filter Paper (Whatman No. 1, W & R Balston, Ltd.).

2) Cellulose Powder (Whatman chromedia CF-11, Reeve Angel Co. N. J.).

3) Microcrystalline cellulose (Avicel PH-101, FMC Corp. Marcus Hook, PA).

4) Carboxymethyl cellulose (CMC-7LP, Hercules powder Co., Wilmington, N. J.).

5) Brown paper towel (Garland, soft-knit, single folder towel, Fort Howard Paper Co.).

6) Spartina alterniflora leaves and stems.

The latter two cellulose sources, i.e., paper towels and Spartina leaves and stems, were treated as follows before subsequent preconditioning. Paper towels were dry, batch shredded in a Waring Blender before pretreatment. Distilled water-washed, dry Spartina leaves and stems were ground in a Wiley Mill (2 mm screen) and passed through a series of screens. The fraction containing particle sizes between 250-500 μ was used in this study.

Substrates were preconditioned using a number of different treatments. As noted, some of these, are used in combination to affect more than one type of cellulose structural parameter, i.e., alkali swelling and mechanical size reduction.

Ball-mill Treatment

Fifty-gram portions of cellulosic sample were added to a 3 liter size porcelain jar with milling stones and placed on a ball-mill apparatus (U.S. Stone ware, Akron, Ohio) for 72 hours at 4 C. Samples were reduced to a flour-like powder with reduced density in liquid suspension.

Alkali Treatment

A cellulose-1% NaOH solution slurry, 1:5 weight/volume, was autoclaved for 10 minutes at 121 C and 15 lbs pressure. The slurry was then filter-washed with distilled water until the pH of the filtrate was near neutrality. After washing, the sample was stored as a 10% slurry in distilled water at 4 C. HCl was used to adjust pH to 7. For incorporation into media, several dry weight determinations indicated that reproducible cellulose quantities could be obtained by pipetting an agitated suspension. Cellulose particles were kept suspended by agitation and rapidly delivered with a large orifice pipette.

U. V. Irradiation

Substrates were irradiated as a paste, 4 g aliquots in 25 ml distilled water or 0.5% $NaNO_2$, spread smoothly over the bottom of an open 150 mm petri plate to a thickness of approximately 1/16 inch. The latter were subjected to U. V. radiation at 4 C for periods of up to 48 hours. Samples were positioned about 6 inches directly underneath the source. The entire irradiation apparatus was enclosed in a box structure to minimize moisture loss to the surrounding air.

The U. V. source was a germicidal lamp (General Electric Co., Bridgeport, Conn.) 18 inches in length and 1 inch in diameter. Ultraviolet output at 253.7nm

was 3.6 watts or 38 microwatts per square centimeter at one meter (Koller, 1965).

Molecular Weight-Degree of Polymerization Study

Molecular weight and degree of polymerization of various cellulose samples were calculated from the intrinsic viscosity of cellulose solutions, using a method described by Swenson (1963).

The cellulose solvent, cadoxen, was prepared from ethylenediamine and cadmium oxide as a stock reagent (Brown and Wikstrom, 1965; Henley, 1960).

Solutions of the cellulose substrates in cadoxen were prepared by dissolving 50 mg cellulose in ice-chilled cadoxen and making up to 10 ml. A 4 ml aliquot of this solution was diluted with an equal volume of 1:1 cadoxen-distilled water, then 4 ml of this diluted solution was charged into an Ostwald viscosimeter and the flow time determined. The viscometer had a flow time for distilled water of 79 sec at 25 C. The solution in the viscosimeter was then diluted repeatedly with equal volumes of 1:1 cadoxen-distilled water, the flow time being determined after each addition of diluent. Decrease in viscosity of the solution at five concentrations was measured at a constant temperature, 25 C, and specific viscosity, n_{sp}, determined using the relationships (Swenson, 1963):

$$n_{sp} = \left(\frac{t}{t_o}\right) - 1 \qquad (1)$$

t = flow time for cellulose solution, in seconds
t_o = flow time for solvent, in seconds

The n_{sp}/C versus C, where C = concentration of cellulose in grams per 100 ml, was then plotted to find the limiting viscosity number, $[n]$. The $[n]$ is the n_{sp}/C value for which C = 0.

According to Brown and Wikstrom (1965), $[n]$ may be used to determine the average molecular weight, $\bar{M}w$, of cellulose in a cadoxen solution by the following equation:

$$[n] = 3.85 \times 10^{-4}(\bar{M}w)^{0.76} \qquad (2)$$

which may be rearranged to the form of equation 3 as shown below:

$$\bar{M}w = (2597.65[n])^{1.3158} \qquad (3)$$

DP was then calculated by dividing $\bar{M}w$ by 162, the molecular weight of the anhydroglucose unit. A sample calculation is presented in Appendix C.

Cellulolysis Determination

In Situ Studies

The methods used for this phase of the investigation were comparable to those described by Hood (1973) and Hofsten and Edberg (1972). Treated or untreated cellulose material, i.e., Whatman No. 1 filter paper squares, Whatman CF-11, or Spartina stem and leaf pieces, were placed in 7 x 7 cm nylon bags which were sewn shut with a tightly folded seam. Fabric forming the bags had a mesh size of 60 microns. Four or five tared bags with known amounts

and placed in a heavy duty nylon bag with a larger mesh size of 0.25 cm. The larger bags were secured with nylon cord to a rod and inserted into a metal trap (Fig. 4). As noted by Hood (1973), the wire trap and heavy duty bags protected the inner bags from crabs and other animals which might have punctured the finer nylon fabric.

The wire trap apparatus was positioned near site 7 in Airplane Lake (Fig. 4 and 5). This site was chosen in view of background information from earlier studies, and because tidal action is notably reduced in the area. A diagram of current patterns and velocities in the west lobe of the lake, taken from Hacker et al. (1970) is given in Figure 6. The current patterns and velocities vary considerably, however, depending upon the prevailing wind conditions. Average salinity in the lake is about 20 parts per thousand (ppt). Ranges in salinity from 18 ppt in the spring to 33 ppt in late fall were recorded by Ho et al. (1970).

Cellulose samples were exposed in bags for a total sample period of January 14 through March 3, 1974. Sample periods are referred to as : I, January 21-26; II, January 27-February 2; III, February 2-10. Temperature data was obtained from the Louisiana State University Coastal Studies Institute.

After appropriate intervals of exposure, the bags were retrieved and returned to the laboratory, in an

Figure 4. Apparatus for in situ Measurements of cellulose degradation Rates. (From Hood, 1973)

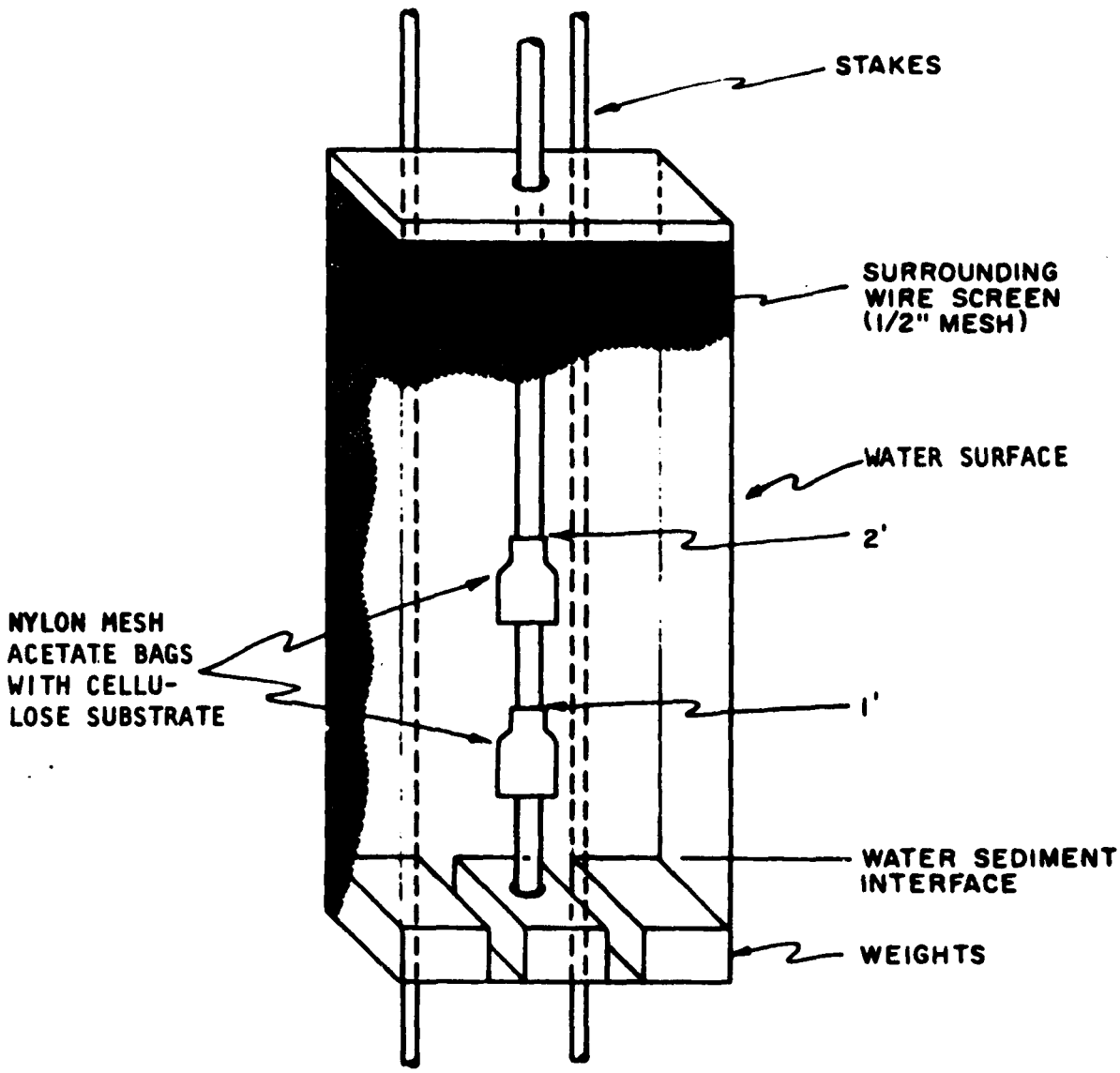

STAKES
SURROUNDING WIRE SCREEN (1/2" MESH)
WATER SURFACE
2'
NYLON MESH ACETATE BAGS WITH CELLU-LOSE SUBSTRATE
1'
WATER SEDIMENT INTERFACE
WEIGHTS

Figure 5. Cellulose degradation apparatus situated in Airplane Lake.

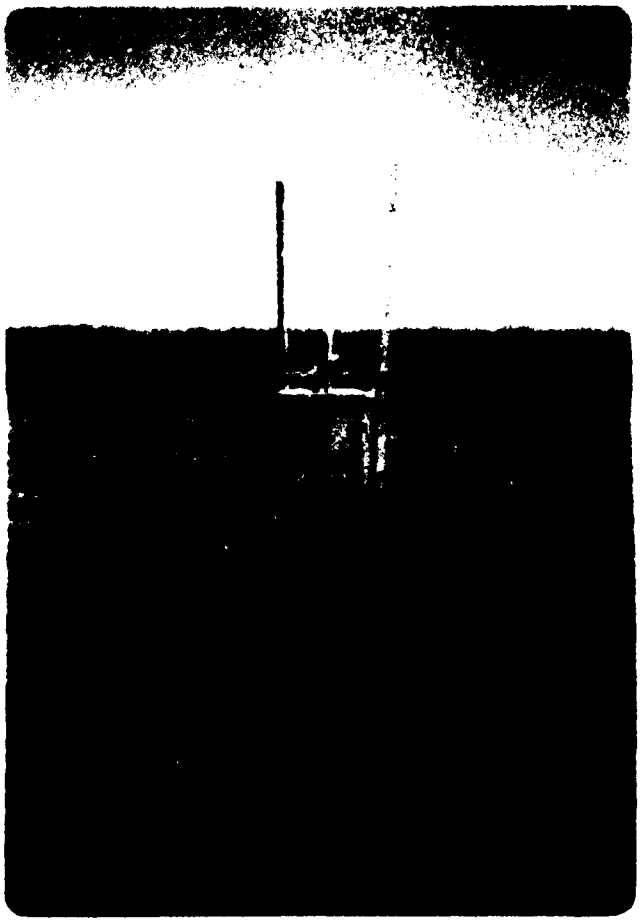
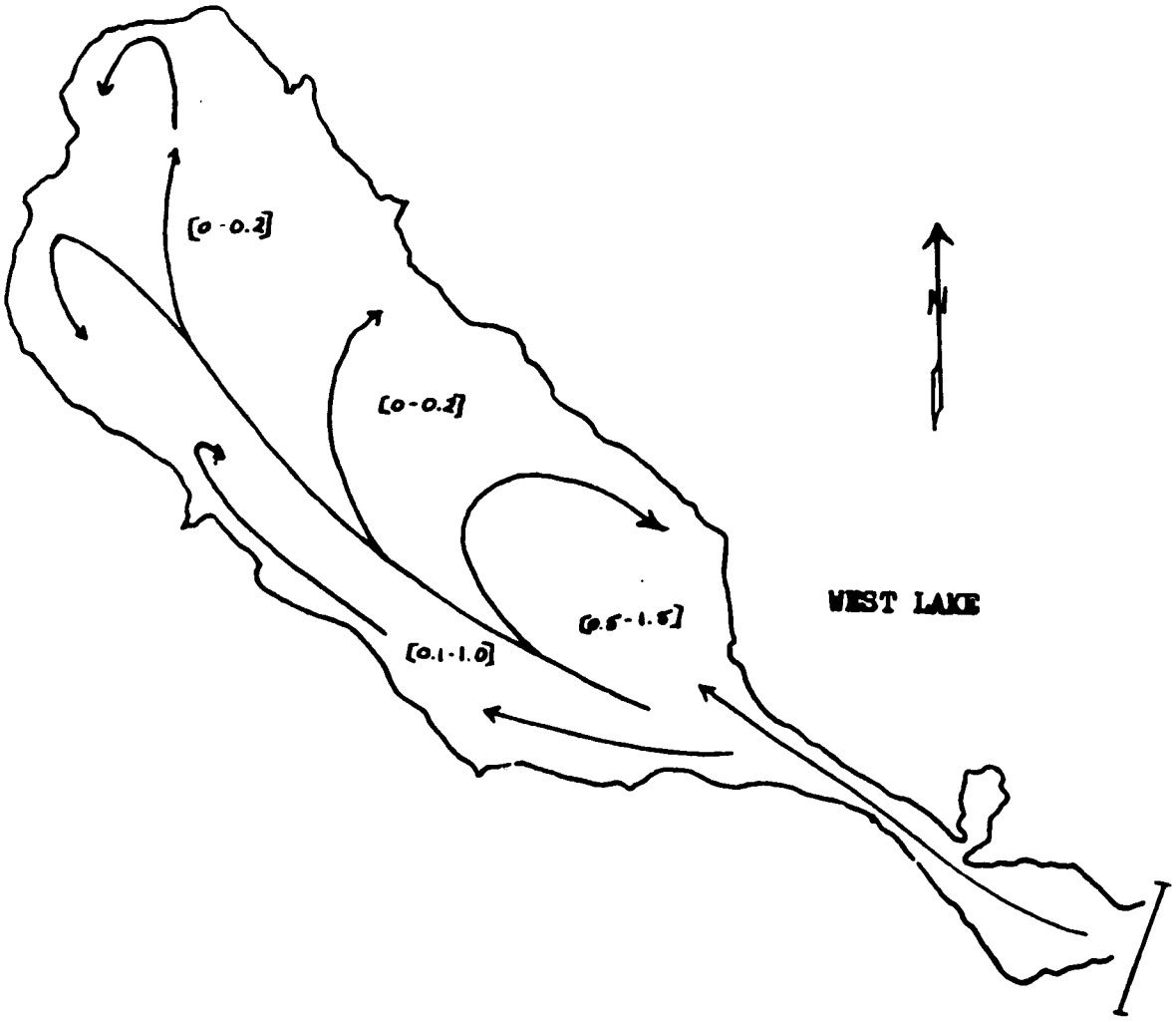


Figure 6. Map of Airplane Lake showing currents

Numbers in brackets indicate velocity (ft/sec.)
(From Hacker, et al., 1970)


[0-0.2]
[0-0.2]
[0.5-1.5]
[0.1-1.0]
N
WEST LAKE

ice chest, within 24 hours for analysis. The bags were first washed with distilled water to remove mud and sediment-accumulated matter. Then, the dip-washing procedure, described below, similar to that reported by Fahraeus (1947) and Lembeck and Colmer (1967), was used to remove cell material. The bags were washed in succession with 12% hydrochloric acid, distilled water, 5% ammonium hydroxide, distilled water, 95% ethyl alcohol, and ethyl ether. Washed bags were then dried to constant weight at 100 C in a vacuum oven, cooled in a desiccator, and weighed. The difference between the initial weight and the remaining cellulose represented the quantity of substrate solubilized per time unit.

Pre-seeded Substrate Studies

To determine the effect of prior inoculation of cellulosic substrates with cellulolytic bacteria, substrates, as noted in the in situ studies, were incubated with selected bacteria. A 24 hr cellulolytic bacterial culture, growing in 300 ml BS with 5% alkali-treated cellulose at 20 C, was harvested by centrifugation, washed twice with 100 ml ANSW. Four or five cellulose bags were then placed in suspension for 12 hrs at room temperature, before exposure in the field.

Laboratory Studies

Degradation studies were performed in the laboratory to facilitate evaluation of the environmental degradation

rates determined. A Gooch crucible-gravimetric method was used in which the amount of substrate remaining, after the action of the cellulose decomposing organism, was a measure of cellulolytic activity. Cellulose samples were placed in 50 ml of BS solution in 250 ml Erlenmeyer flasks and autoclaved before inoculation with 1 ml of 24 hr culture of the cellulolytic bacteria grown as described above in the Preseeded Substrate section. The flasks were incubated at 30 C on a rotary shaker at 150 rpm. At intervals, flasks were removed and contents filtered in Gooch crucibles (Pyrex fritted glass, 30 ml capacity, M-porosity). The method of Lembeck and Colmer, (1967) described above, i.e., 12% hydrochloric acid, 5% ammonium hydroxide, ethyl alcohol, and ethyl ether, was used as a washing procedure. The crucibles were dried to constant weight at 100 C in a vacuum oven and cooled in a desiccator. Amounts of substrate solubilized were determined by difference in weight.

Polychlorinated Biphenyls

Paper towels and prepared cellulose samples were analyzed for the presence of PCB residues according to the methods of Dube et al. (1974) and Senum et al. (1973) using electron capture gas chromatography.

Samples With Known Contamination

A 50 gm portion of petroleum ether washed Whatman CF-11 cellulose powder was prepared to contain 2 ppm

of the PCB, Aroclor 1260 (Analabs, Inc. North Haven, Conn.). Uniform distribution of the PCB was obtained by tumble drying the cellulose powder with a known quantity of Aroclor 1260 in a petroleum ether solution. Portions of the prepared sample were then treated as follows to determine if PCB residues could be affected:

1) U.V. irradiation of dry powder up to 48 hrs.

2) U.V. irradiation up to 48 hrs for a slurry of 4 g powder in 50 ml distilled water.

3) U.V. irradiation up to 48 hrs for a slurry of 4 g powder in 50 ml 0.1% sodium nitrite solution.

4) Autoclave 10 min. at 121 C. A slurry of 4 g powder in 50 ml 1% sodium hydroxide solution was used.

5) Control samples:
   a. 4 g dry powder
   b. 4 g powder slurry in 50 ml distilled water.

Unknown Samples

Brown paper towels, as a typical commercial cellulosic product, were examined for the presence of electron capture peaks. Other workers (Senum et al., 1973) have reported electron capture peaks from extracts of paper towels. Further characterization of the peaks showed that they resulted primarily from PCB compounds and not from other possible chlorinated compound residues. Shredded towel (described in the Substrate Preparation Section) was

analyzed after the following treatments:

1) U.V. irradiation up to 48 hrs for 4 g paper with 50 ml distilled water.

2) Autoclave for 10 min. at 121 C, slurry of 4 g paper in 50 ml 1% sodium hydroxide solution.

3) Control samples: a. 4 g dry powder
b. 4 g powder in 50 ml distilled water

The fate of electron capturing residues during bacterial cellulolysis of paper was investigated by inoculating paper towel substrate with the cellulolytic isolate, cm. Erlenmeyer flasks, 250 ml, containing 100 ml BS solution with 4 g shredded brown paper towel were autoclaved and then inoculated with 1 ml of 24 hr cellulolytic culture, actively growing isolate Cm, prepared as in the Laboratory Studies Section. Flasks were incubated 5 days at 30 C on a rotary shaker, following which the cellulose cultures were centrifuged at 7,000 rpm for 10 min. and decanted. The supernatant broth was retained for analysis. The pellet was washed twice with cold, BS solution and then extracted for gas chromatography.

Extraction and Cleanup of Samples

One of two extraction procedures were used, depending on whether the sample was wet or dry.

Dry sample extraction--

1) A 4 g sample was placed in a 250 ml separatory funnel and shaken vigorously with 100 ml hexane.

2) The hexane was carefully decanted and retained.

3) Steps 1 and 2 were repeated and extracts combined and concentrated to 10 ml for cleanup.

Wet sample extraction--

1) Slurries (4 g dry weight in 50 ml liquid) or 50 ml liquid samples were placed in a 500 ml separatory funnel.

2) Fifty milliliters acetonitrile were added and the funnel shaken intermittently for 10 minutes.

3) One hundred ml hexane were added and the flask vigorously shaken. After the layers separated, the hexane layer was decanted and retained.

4) Step 3 was repeated and the extracts combined.

5) The combined hexane extracts were shaken twice with 2% anhydrous, petroleum ether washed, sodium sulfate granules.

6) Extracts were concentrated to 10 ml for cleanup.

Cleanup procedure--Sample cleanup was done by means of liquid-solid chromatography with florisil, activated at 105 C. The florisil column used was a 22 mm (ID)X 250 mm glass column with a 300 ml capacity. First, approximately 0.5 in of anhydrous sodium sulfate was added. Then 4 in of activated florisil were added while tapping the outside of the column to insure that the florisil was firmly packed. One-half inch anhydrous sodium sulfate was added to the top of the column to absorb any moisture from the hexane extract.

To wet the prepared florisil column, 50 ml of hexane was added and eluted to the top of the packing surface. The appropriate amount of sample extract was then added. As the sample solution reached the packing surface, 50 ml of hexane was added and elution rate adjusted to 3 to 5 ml/min while collecting the eluant. After the first 50 ml of eluting solvent was collected, an additional 150 ml hexane was added to the top of the column and collected.

The eluant from the cleanup procedure was evaporated with a Rotavapor (Buchi, Inc., Switzerland) flash evaporator at 45 C. After concentration to approximately 2 ml, the sample was transferred to a volumetric flask and diluted to a known volume with hexane. Appropriate further dilutions were made for analysis by electron-capture gas chromatography.

Gas Chromatograph Parameters

Gas chromatographic analyses were conducted on a Varian Aerograph 1200 instrument with an electron-capture detector. Table 2 gives a summary of the conditions of operation.

Three to five microliters of the sample were injected for analysis. Sample peak heights were compared with peak heights of known amounts of PCB standards (Aroclor 1254, 1260-Analabs, Inc., North Haven, Conn.) in order to obtain sample concentrations. Peak heights

TABLE 2

Operational Parameters for Gas Chromatographic Analysis

Instrument----------Varian Aerograph 1200

Component and Mode of Operation	
Detector	Electron capture
Source	Tritium
Activity	150 mCi
Applied Voltage	90 volts
Temperature	205°C
Column	Pyrex
Diameter	1/8 in.
Length	5 ft.
Packing	
Solid Support	Chromosorb W
Liquid Phase	10% DC-200
Temperature	185°C
Carrier Gas	Nitrogen
Flow Rate	64 ml/min.
Injector	225°C

were obtained by drawing the best base line under the peak and measuring from the base line vertically to the center of the peak.

Preliminary identification of unknown sample residues was made by comparing chromatograms of standard Aroclors with the sample chromatograms to determine which Aroclor the sample most resembled. An estimate of the concentration present was made by measuring the heights of the most prominent sample peaks that coincided with prominent peaks of Aroclor standards.

RESULTS AND DISCUSSION

Studies by Hood (1970) indicated that cellulolytic bacterial populations in Airplane Lake sediment and water are rather constant. An MPN-filter paper strip method was used to enumerate cellulolytics. MPN-counts per gram wet weight of sample, showed 10^3 to 10^4 cellulose decomposers in sediment, and higher levels of 10^7 to 10^8 organisms in marsh soil. Water samples contained an average of 10^3 cellulolytics per milliliter, which represent approximately from 1 to 10% of the total heterotrophic aerobic bacterial population.

Crow (1974) found slightly lower cellulolytic population levels as measured by a CMC-viscosimetric technique. Sediment samples from Airplane Lake contained from 10^5 to 10^6 cellulose decomposers per gram wet weight.

Both of the aforementioned studies indicated a slight increase in numbers of cellulolytics during the spring months of February to April, in 1970 and 1973. Thus, the isolation and degradation studies of this investigation were carried out during probable peak periods of cellulose degradative activity concurrent with maximal cellulolytic bacteria populations.

Cellulolytic Isolates

In many instances, enrichment tubes or flasks initially evidenced cellulose degradation, however,

cellulolytic activity was lost upon subsequent transfer of the enriched broth to fresh enrichment tubes. Similarly, after a few weeks of laboratory maintenance, isolated cultures frequently lost their ability to degrade cellulose. Loss of cellulolytic activity after laboratory maintenance of cultures has been encountered in several studies by other workers (Crow, 1974; Chahal and Gray, 1969). Chahal and Gray (1969) speculated that perhaps loss of activity was due to depletion of endogenous cellulase cofactors which were present in native cellulosic substrates.

Sixteen isolates are enumerated in Table 3. All of the bacteria consistently showed cellulose degradation on filter paper in basal salts solution. However, certain of the isolates varied in their ability to produce clearing zones when grown on cellulose-agar overlay plates. Kadota (1956) noted that formation of enzymatic areas of cellulose decomposition around colonies did not accompany growth of Cytophaga or certain Vibrio species. Colonies of Cytophaga showed clearing of cellulose only directly beneath the growth area. This seems to indicate that direct bacteria-cellulose contact is necessary for degradation of the polymer and that a non-diffusible or membrane bound cellulase is involved. A study of extracellular and cell-bound cellulases of bacteria has been discussed by Suzuki et al. (1969). Their results show that both cell-bound and extracellular cellulases may be produced by an organism and that relative distribution

TABLE 3

Selected Characteristics of Cellulolytic Isolates

Isolate	Number of Isolations	Cell Shape	Gram Reaction	Pigment*	Morphology*	Clearing on Cellulose-overlay agar	Source of Isolation
JB-1	1	rod	-	yellow	small, butyrous	+	MP**
JB-2	1	short rod	-	white	small, butyrous	+	Spartina detritus
JB-3	2	rod	-	yellow	umbonate, translucent	+	Spartina detritus
JB-4	1	rod	-	clear	small, translucent	+	Marsh sediment
JB-5	1	curved rod	-	yellow-orange	high standing	-	Marsh sediment
JB-6	3	short rod	-	white	small	+	Marsh sediment
JB-7	1	curved rod	-	brownish	spreading	-	MP
JB-8	2	short rod	-	yellow	translucent	-	Water
JB-9	1	rod	-	white	translucent	-	Marsh sediment
JB-10	1	rod	-	yellow	fish-eye	-	Water
JB-11	1	curved rod	-	yellow	spreading	+	Water
JB-12	3	rod	-	white	translucent, spreading	+	Spartina detritus
JB-13	2	short rod	-	clear	small	-	Marsh sediment
JB-14	1	rod	-	brownish	translucent	-	MP
JB-15	2	curved rod	-	orange	thin, spreading	+	Spartina detritus
JB-16	1	rod	-	clear	umbonate	-	Water

*Colonial morphology and pigment production on Marine Agar 2216 (Difco).

**MP = Martigan Point, island in Barataria Bay.

of endo or exo-cellulase may vary with different substrates.

According to Kadota (1956), organisms such as Vibrio purpureus or V. aquamarinus showed no zones of enzymatic hydrolysis either around or beneath the colonies. Yet, both species rapidly macerated filter paper. Similar action was noted in this study, for isolates JB-5, JB-7, JB-8, etc., and may indicate that the mode of cellulose decomposition by marine aerobic cellulolytics is species dependent. Presence of C_x cellulase components only, might allow degradation of amorphous cellulose regions and cause fiber disaggregation while leaving the major crystalline fiber matrix intact.

After isolation procedures, the organisms Cm, JB-1, and JB-3 were selected for further study of their cellulolytic action. Isolate Cm, obtained from M. Fleenor, Louisiana State University Department of Microbiology, has been shown to decompose a wide variety of cellulosic substrates (Han and Srinivasan, 1968) and is currently under consideration for use as a cellulase producer in the LSU-SCP program (Srinivasan, 1974). The organism was found to grow readily and decompose cellulose with little inhibition in sea water. However, colony size and pigmentation were not as great on Marine Agar (MA) or Basal Salts Agar (BSA) as they were on Nutrient agar or BSA with no NaCl added.

An interesting aspect in the isolation of JB-1 is that no Cellulomonas species have been reported from marine environments. However, Martigan Point, the isolation

site, had received oil following a pipeline break in Barataria Pass approximately 6 months previous to the isolation. In an attempt to contain the major portion of oil upon the island, ground corncob and straw were liberally spread over the oil. Later, this weathered cellulosic material mixed with Spartina detritus was used in this study as an isolation source for cellulolytic bacteria. Thus, the isolate JB-1 is quite likely of terrestrial origin rather than an indigenous marine cellulose decomposer.

Both Cm and JB-1 rapidly (within 3 days) macerated filter paper in basal salts solution with extensive solubilization evident after 5 days. If a filter paper tube was left unshaken, the paper strip became translucent and then separated at the air-water interface. Isolate JB-3 represented another genus, Vibrio, and had a macerative action on filter paper without complete solubilization. In contrast to the former two isolates, the action of JB-3 caused fiber disaggregation which resulted in a pulpy macerated mass without complete solution of the substrate. All three organisms produced colonies surrounded by cleared zones on cellulose-overlay agar. Table 4 shows a partial characterization of Cm, JB-1, and JB-3. As in other marine studies (Alexander, 1973, Hood, 1970), only gram negative rods were found in the isolates having cellulolytic activity. Several times in this study. cellulose-decomposing colonies were isolated which proved

TABLE 4

Characterization of Selected Cellulose-Utilizing Organisms

	Isolate		
	Cm*	JB-1	JB-3
Morphological Characteristics:			
Form	rod	rod	curved rod
Size	.5μ x 1.0-1.5μ	.4-.7μ x 1.0-1.5μ	.3μ x 1.0-2.0μ
Motility	non-motile	motile	motile
Flagella	--	peritrichous	polar
Gram stain	−	−	−
Biochemical Characteristics:			
Gelatin stab	slow liquefaction	slow liquefaction	no growth
Milk agar (proteolysis)	--	--	--
Glucose O/F	F	F	F
Starch	hydrolyzed	hydrolyzed	hydrolyzed
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Cellobiose	+	+	+
Xylose	+	+	+
Fructose	+	+	-
Galactose	+	+	-
Action on filter paper:	lytic	lytic	macerative
Genus	Cellumonas	Cellumonas	Vibrio

*Organism obtained from M. Fleenor, Department of Microbiology, Louisiana State University, Baton Rouge (Han and Srinivasan, 1968).

to be gram positive yeast phases of cellulolytic filamentous fungi. However, only cellulolytic bacteria were used in these experiments.

Cellulose Agar Isolation and Enumeration

The cellulose agar-overlay technique worked quite well in isolation procedures. Colonies from plates containing less than 20 to 30 colonies could be picked easily and restreaked to fresh media. When more than 30 colonies were present on one plate, overgrowth and cross-contamination of colonies resulted in poor separation of cellulolytic isolates. The overlay procedure was also used in several enumeration attempts. When low total populations of bacteria were present, the cellulose agar system could be used for enumeration, with a recognition that some cellulolytics do not produce cleared zones. Table 5 shows that the procedure may be used to indicate that there are few cellulolytics present in open sea water. Numbers of total heterotrophic aerobes, 10^4/ml, are also shown to be relatively low in contrast to total counts of 10^5-10^6 organisms/ml in Airplane Lake. The low numbers of cellulolytic bacteria in deep-sea samples was further confirmed by the lack of cellulose degradative activity in enrichment tubes. Open sea samples were very transparent with little suspended or precipitated particulate matter. These findings support the suggestion of Floodgate (1966), Seki (1968, 1972), and others, that most bacteria in the ocean more or less attach themselves

TABLE 5

Enumeration of Cellulolytic Organisms
from Deep Water Sampling Site

	Average Number of Organisms/ml	
	Water from Surface	Water from One Meter
Total plate count (Marine Agar 2216)	3.7×10^2	2.8×10^2
Cellulolytics before enrichment*	2	0
Cellulolytics after enrichment*	0	0

*Cellulose overlay agar plates were used. No cellulolytic activity was noted in enrichment tubes. Counts represent average values for 5 replicate samples at each water level.

to a surface and exist in association with particulate matter of aggregates. Thus, where there is little particulate material present, one could expect low bacterial populations. Similarly, one would expect particulate matter to become associated with a high bacterial biomass. Relating this to cellulose decomposition, it might correctly be assumed that cellulolysis would occur very slowly in the open sea since the level of cellulolytic organisms is low. Intuitively, then, the process could be accelerated by somehow increasing the chance of interaction of cellulosic particles and organisms capable of degrading them. Jannasch and Wirsen (1973) found negligible decomposition of paper and wood samples after 1 year at 1830 meters in the open sea. However, they indicated that in a particulate form these substrates might never have reached the depth studied. An estimated sedimentation rate of from several weeks to more than a year per 1000 meters of depth would allow extensive degradative processes to occur if a suitable microbial flora were established. Their study also indicates that very little is known about the quantity or recycling of nonrefractory organic matter which reaches the deep-sea floor.

Degree of Polymerization Studies

Pretreatment of cellulosic substrates by various procedures produced a lowering in degree of polymerization

(D.P.) as indicated by Table 6. The ball-milling pre-treatment was used with only the intention of increasing the suspendability of cellulose powder in a cellulose-agar isolation medium. However, reduction in both size and D.P. were achieved.

Alkali treatment resulted in a slight decrease in D.P. which is consistent with results reported by Richards (1963). The latter notes that alkali treatment of celluloses having a D.P. distribution resulting from random chain scission (as in Whatman CF-11 cellulose powder) may produce negligible effects on the D.P. of the residual cellulose. The primary action of alkali appears to be in alteration of the crystallinity of cellulose fibers concurrent with absorption of water. Han and Callihan (1974) found very little carbohydrate solubilized by alkali treatment of cellulose and stated that increased bacterial susceptability of fibers was due to structural change within the crystalline matrix.

The degree to which alkali treatment swells cellulose fibers may be noted in Figure 7. The photomicrographs show alkali-swollen fibers compared with non-treated fibers of CF-11. Surfaces of control fibers appear smooth, while treated, swollen, fibers are ridged and quite open in structure.

Ultra-violet irradiation of CF-11 produced a striking decrease in D.P. As suggested by Fookson and Frohnsdorff (1973), incorporation of 0.5% sodium nitrite

TABLE 6

Degree of Polymerization of Pretreated Cellulosic Substrates

Substrate	Pretreatment	$\bar{M}w$*	D.P.
Whatman CF-11	None	108,800	672
Cf-11	Ball-mill	69,900	432
CF-11	Alkali	85,000	525
CF-11	48 hr U.V. with dist. water	96,170	594
CF-11	48 hr U.V. with $NaNO_2$.	63,100	390
Avicel PH-101	None	77,450	478

*$\bar{M}w$-average molecular weight. (As described in Appendix C).

Figure 7. Swelling action of alkali treatment on cellulose fibers. Smooth-surfaced fibers are untreated (A); wide, ridge-surfaced fibers are alkali treated (B).

into the irradiation slurry greatly increased the rate of depolymerization. The total degree of depolymerization was increased by a factor of slightly more than 1.5. These results are presented in Figure 8. Both samples, with and without nitrite, showed a rapid drop in D.P. followed by a gradual plateau after 24 hrs of irradiation. Continued U.V. treatment produced little further decrease in levels of polymerization.

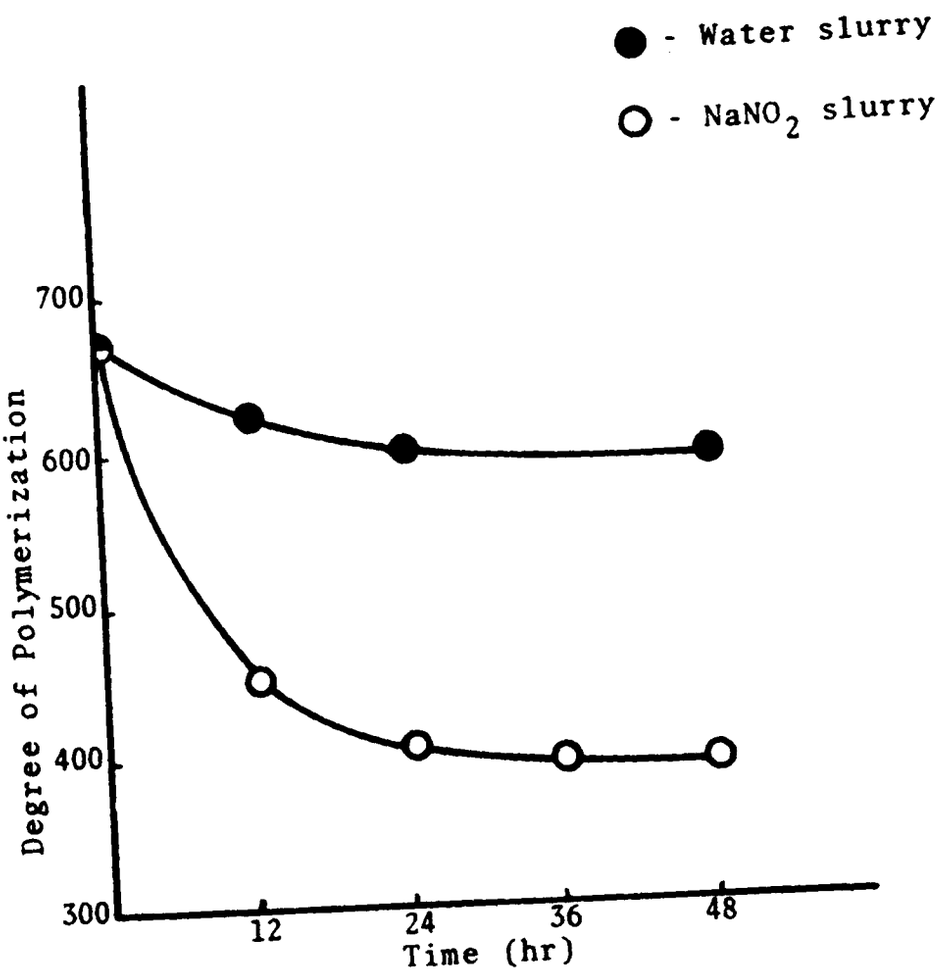
In Situ Cellulose Degradation

Solubilization rates for various cellulosic substrates were determined in a marine environment. As previously noted, Hood (1973) determined degradation rates for chitin and Hofsten and Edberg (1972) estimated rates for cellulose fiber degradation in marine environments using in-situ methods. However, other than the latter study, no quantitative determinations of cellulose decomposition in a marine environment were found in a review of the literature of cellulose degradative activities.

Measurement Considerations

The "bag" assay method used in this study requires that certain assumptions be made if one is to correlate weight losses of bags containing cellulose fibers with rates of degradation of similar fibers in surrounding waters. Hofsten and Edberg (1972) have discussed several of these assumptions. First, conditions inside the bag must approximate those outside. This requirement is most

Figure 8. Effect of 0.5% sodium nitrite on depolymerization of CF-11 cellulose powder by U.V. irradiation.


● - Water slurry
○ - $NaNO_2$ slurry
Degree of Polymerization
700
600
500
400
300
12
24
36
48
Time (hr)

probably fulfilled because of the relative high porosity and small size of the bags. Fibers inside the bag are exposed to the same microbial populations, temperatures, and nutrient conditions as would be external fibers.

Second, growth of benthic organisms on and within the bags should be negligible. Within the time period of this study, the nylon bag fabric remained inert. Microscopic examination of fabric fibers showed that diatoms were present, however their residue weight after acid-alkali washing was very low. Empty control bags were included in all experiments to indicate any non-cellulosic weight changes. The control bags showed very little weight change. Figure 9 indicates the degree of growth that may occur upon the bags during extremely long exposure. The bag had been exposed for 46 days and had quite extensive Crustacean (barnacle) and Bryozoan colonization. Early development of these organisms was just beginning when the last samples (33 days) of this study were removed from the study area. Hofsten and Edberg (1972) noted a weight increase, particularly in summer months, in some of their samples, which they attributed to growth of algae on and within the bags. They found that the algal growths were difficult to remove in a reproducible manner. Problems with growth of algae were not encountered in the present study.

Figure 9. Cellulose assay bag and protective outer mesh after 46 days environmental exposure.

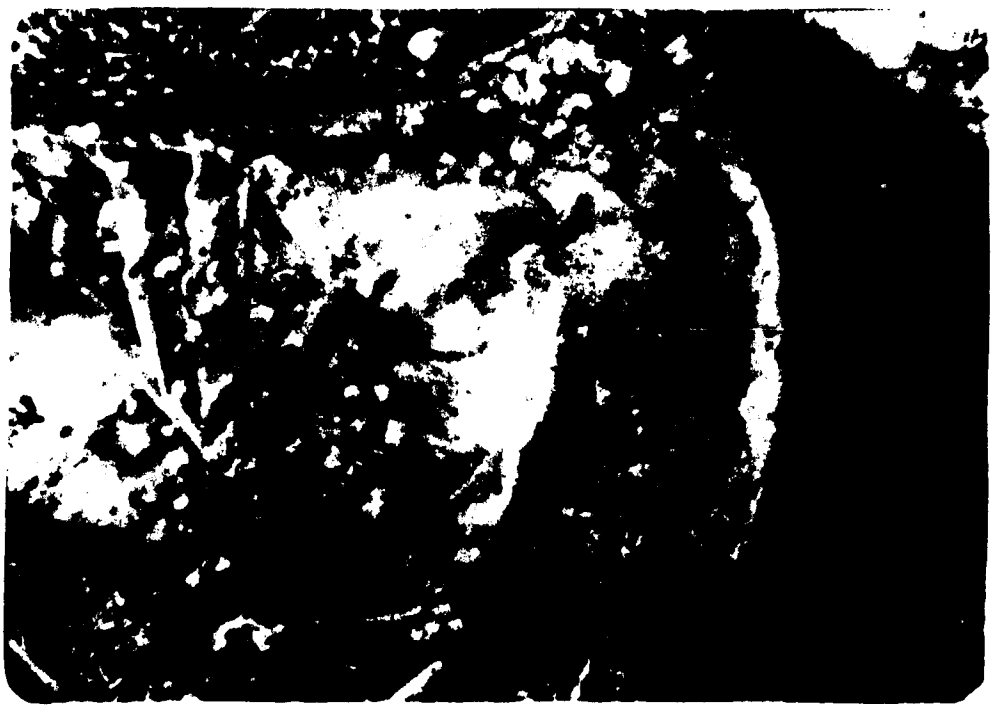
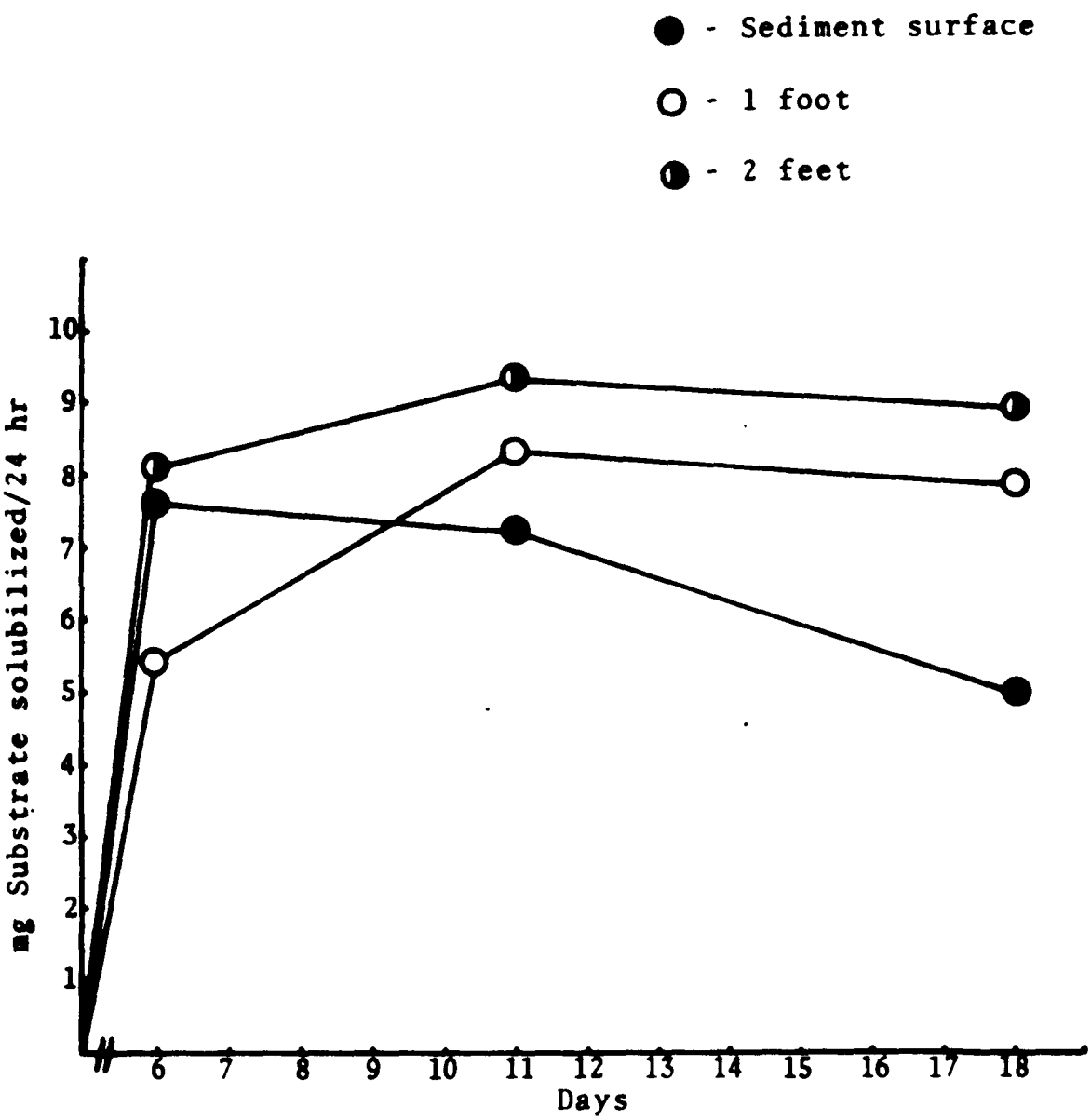


Figure 10. Cellulose solubilized at sediment-water interface, 1, and 2 feet. Filter paper was used as substrate.


● - Sediment surface
○ - 1 foot
◐ - 2 feet
mg Substrate solubilized/24 hr
10
9
8
7
6
5
4
3
2
1
6
7
8
9
10
11
12
13
14
15
16
17
18
Days

Degradation Rates

The highest rates of cellulose decomposition occurred at a water depth 2 feet above the water-sediment interface. Figure 10 indicates results from the findings of Hood (1973) for chitin degradation in which decomposition rates were highest at the sediment-water interface and lowest in the water column. In laboratory studies, with filter paper strip-test tube cultures of cellulolytic bacteria, degradation always occurred first at the air-water interface. Throughout most of this study the 2-foot level (see Methods section) of the assay apparatus was just below the surface of the shallow estuarine lake, i.e., the air-water interface. Thus, it would appear that a high degree of oxygenation is requisite for most rapid cellulose solubilization. Hofsten and Edberg (1973) found very low cellulolytic activity in oxygen depleted water of eutrophic lakes.

Solubilization rate at the sediment-water interface peaked slightly more rapidly than did the rate of cellulolysis at 1 foot above the sediment. This may indicate a more rapid substrate colonization by cellulolytic organisms at both the sediment and water surface. An area of further research might include enumeration of cellulolytic organisms in the neuston. The importance of the top few millimeters of sea surface is just beginning to be realized. MacIntyre (1974) has discussed recent

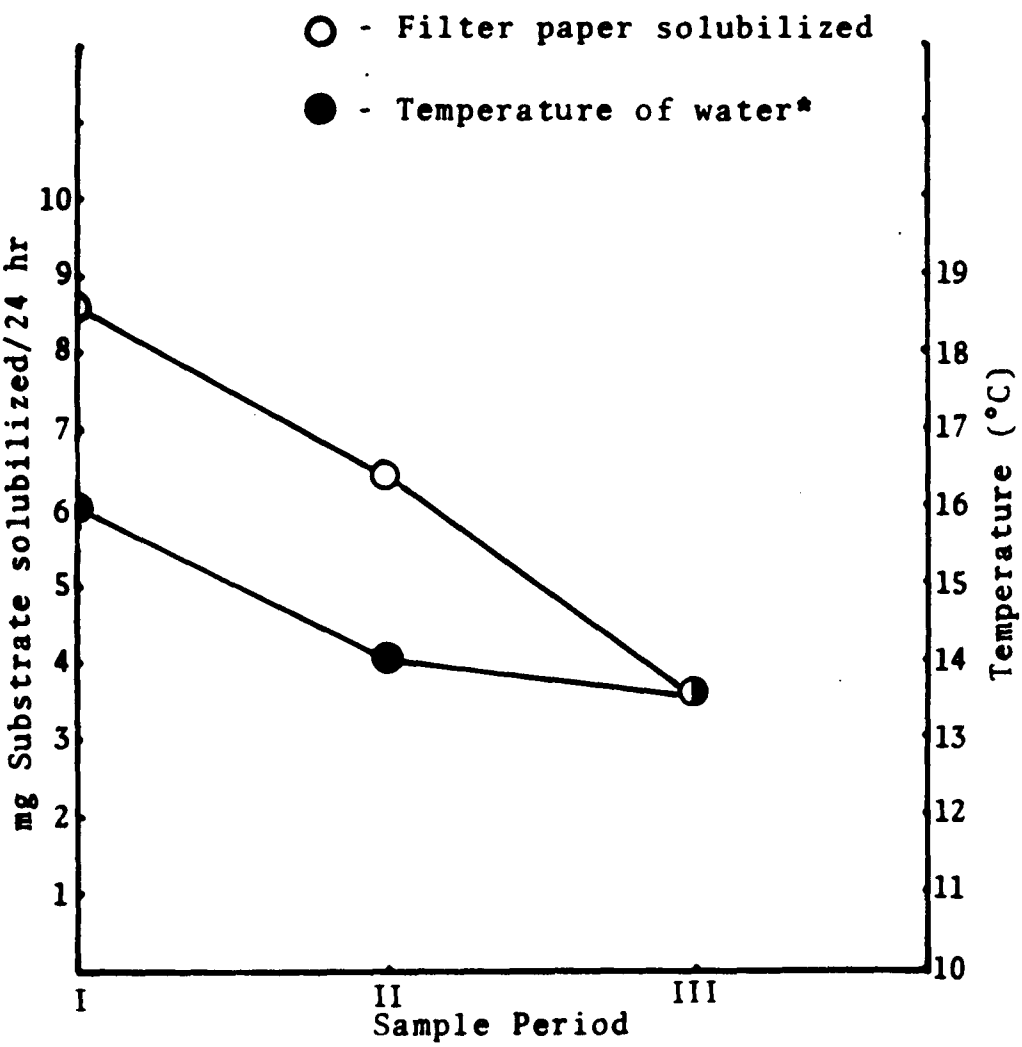
research concerning biological and physical interactions in this area. Benthic levels of cellulolytic population have been previously examined in studies by Hood (1970) and Crow (1974) and indicate that numbers of cellulolytics are higher at the sediment-water interface than in the water column.

Investigation of the photolysis of recalcitrant compounds, i.e., DDT, PCB, etc., within the surface layer, appears especially warranted, concurrent with examining adsorptive phenomena of these toxicants within detrital transformation systems. Preliminary experiments in this study indicate that surface microbial populations, both total and cellulolytic, may exceed levels at the sediment-water interface. Metabolic activity within the surface layer, however, has not been examined. Therefore, further work is necessary to determine if the high bacterial population is a consequence of physical factors and whether it is indicative of microbial metabolic activity within the surface layer.

Temperature medians for surface waters at the mouth of Airplane Lake were 15 and 16 C for the study months of January and February, respectively (Hopkinson, 1974). Figure 11 shows the effect temperature variation may have on decomposition rates. These results are consistent with the findings of Laurent (1969) for cellulolysis in mud, and Hood (1973) for chitin degradation. Table 7 also indicates decreased cellulolytic

Figure 11. Correlation of mean temperature of water with in situ cellulose degradation rate (1 foot sample).

Water temperatures at the mouth of Airplane Lake were obtained from Louisiana State University Marine Science Department (Hopkinson, 1974). See Methods section.


○ - Filter paper solubilized
● - Temperature of water*
mg Substrate solubilized/24 hr
Temperature (°C)
10
9
8
7
6
5
4
3
2
1
19
18
17
16
15
14
13
12
11
10
I
II
III
Sample Period

activity corresponding to progressively lower temperatures during the sampling periods. All of the values except that for the 2-foot filter paper sample show a decrease in rate. Perhaps localized daytime surface warming served to maintain the higher decomposition rate of surface samples.

Figure 12 shows decomposition rates for a "natural" versus an introduced cellulose source. Processed cellulose, filter paper, was degraded relatively slowly compared to the indigenous Spartina substrate. Several studies have examined the importance and mode of microbial decomposition of marshgrass (Udell et al., 1969; Burkholder and Bornside, 1957; and Zieman, 1968). Udell et al. (1969) reported that little herbivorous grazing occurred in standing Spartina and that major decomposition routes follow bacteria-rich detritus formation. The efficiency of decomposition mechanisms must be quite high since about 55% of the net marsh production (as high as 14 tons per acre/year according to Odum, 1959) is dissipated by indigenous consumers, while 45% is transported away from the production area (Teal, 1962). Zieman (1968) found that drying of Spartina appeared to decrease Spartina degradation time by disrupting the tissue cuticle and making the grass more porous and accessible to bacteria.

A number of explanations might account for relatively greater "natural" substrate degradation. The resident marsh microbial population may be more specifically

Figure 12. In situ degradation of "Natural" vs introduced substrate at a water level 2 feet above the sediment-water interface. Rates are indicated as mg substrate solubilized/24hrs/gm substrate.

adapted in its enzyme complement to solubilize and recycle Spartina detritus components. Or, noncellulosic compounds in the detritus may act as positive chemotactic stimuli for cellulolytic organisms. The latter explanation may also account for the early peaking and gradual decrease in rate of decomposition of Spartina. More readily available organics, which could act as cellulolytic attractants (Mitchell, 1972) might be utilized first in the decomposition process followed by a slower, diauxic, utilization of cellulose. This explanation appears to be well-grounded when one considers that Spartina may average 14% protein and from 1 to 3% lipid (Burkholder, 1956). Kadota (1956) and others have noted decreasing cellulolysis with time as a function of decreasing amounts of amorphous cellulose concurrent with increasing amounts of crystalline material in the residual substrate matrix. Thus, as more easily degraded amorphous cellulose is solubilized, the remaining crystalline fiber structure becomes increasingly resistant to cellulolytic attack.

Effect of Substrate Pretreatment

Numerous studies have been noted in which substrate pretreatment by various means has been used to accelerate laboratory decomposition of cellulosic materials (Han and Callihan, 1974; Tyagi, 1972; etc.). Two of the more effective pretreatments noted were alkali treatment and U.V.

irradiation in the presence of sodium nitrite. Figure 13 shows the effect of these pretreatments singly and in combination. Both alkali and U.V. treatments increased degradation rates by factors of 4.8 and 3.7, respectively. The results of U.V. photolysis were supported by the findings of Fookson and Frohnsdorff (1973). They reported that although some increase in degradation rate was achieved through irradiation, (5 to 7% increase) greater increases (more than 20%) resulted from alkali pretreatment. Their study concluded that U.V. irradiation, with or without nitrite, was not a practical treatment for accelerating cellulose decomposition rates. It may be noted, however, that a combined U.V. and alkali, exposure, with bacterial seeding, (Figure 13) gave higher solubilization rates than did either pretreatment by itself.

Fleenor (1973) reported that the cellulolytic isolate, Cm, affixes itself to cellulose fibers during the process of cellulose degradation. The mechanism of this attachment has not been elucidated. In this study, an attempt was made to pre-seed cellulose with a sessile-conglutinating cellulolytic bacteria population, i.e., isolate Cm or JB-1. The results are presented in Figure 13 and Table 8. Solubilization rates of seeded substrates were increased by a factor of 2 compared with values from non-seeded paper and Spartina

Figure 13. Effect of substrate pretreatment on in situ degradation rates. Two foot level.

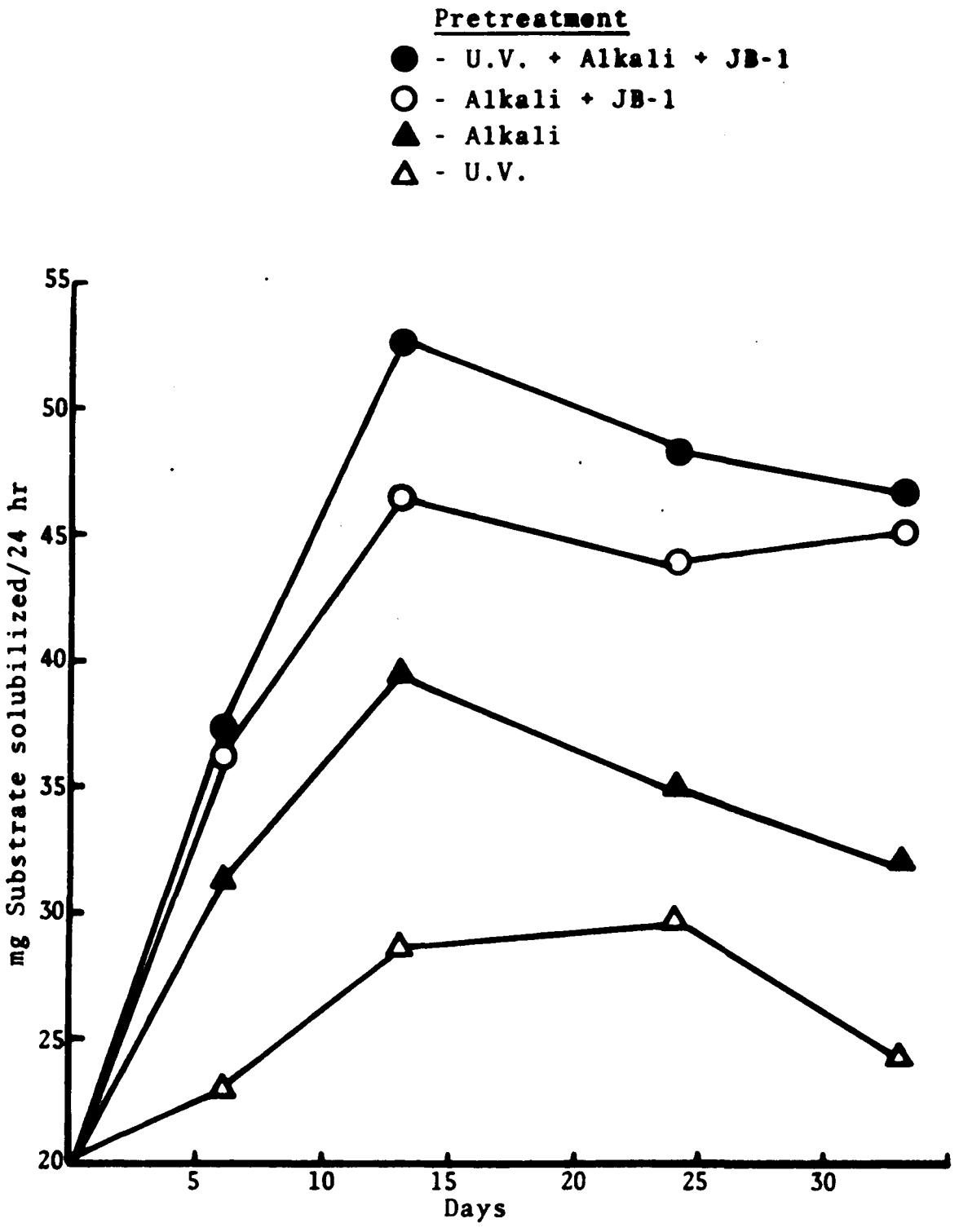

Pretreatment
● - U.V. + Alkali + JB-1
○ - Alkali + JB-1
▲ - Alkali
△ - U.V.
mg Substrate solubilized/24 hr
55
50
45
40
35
30
25
20
5
10
15
20
25
30
Days

TABLE 8

In Situ Solubilization Rates* of Pre-seeded Cellulosic Substrates. Pre-seeding Organisms were Isolates JB-1 and Cm Two foot level.

	Substrate	
Seeding Organism	Filter Paper	Spartina
None	6.7	36.8
JB-1	13.0	57.0
Cm	11.5	49.2

*Rates are shown as mg substrate solubilized/24 hrs.

degradation. It is also interesting to note that the isolate JB-1, from a marine environment, showed slightly higher cellulolytic activity in pre-seeding experiments than did the vigorous Cm isolate. Perhaps this indicates some degree of adaptive ability of Cellulomonas sp. to a marine environment. Bacterial pre-seeding of cellulose, U.V.- and alkali-conditioned, increased its rate of decomposition by a factor of 6.4. By accelerating the rate of decomposition to such a degree, one conceivably also could increase the total degradative capacity of an ecosystem in which cellulose was a major component. This consideration could be of particular consequence to the ecology of ecosystems which are continually stressed by vast influxes of cellulosic waste material. Hofsten and Edberg (1972) reported widespread, deep, cellulose fiber deposits in the recipient water of many pulp and paper factories.

Table 9 summarizes the results obtained by in situ decomposition experiments. The low rate of 3.8 mg substrate solubilized per 24 hr per gram of substrate, for untreated filter paper, compares with relatively low solubilization rates for chitin, i.e., 4.6 mg/24hrs/gm chitin, in deep coastal sediments (Liston et al., 1965). Approximate values for in situ cellulolysis rates were calculated from the limited data presented by Hofsten and Edberg (1972) (Table 10). The values shown in Table 10 compare favorably with in situ rates determined

TABLE 9

In Situ Solubilization Rates of Various Cellulosic Substrates. Pretreatment of Substrate is Noted. Degradation rates are presented as mg Substrate Solubilized/24 hr except where noted. Two-foot level.

	Substrate	Days 6	13	24	33	Mean Rate	Mean Rate per Gram Substrate in Sample
1)	Filter Paper	5.7	8.2	7.0	7.8	7.2	3.8
2)	Alkali Filter Paper	31.3	39.5	35.0	32.1	34.5	18.2
3)	U.V Irradiated Filter Paper	23.0	28.7	29.9	24.2	26.5	14.2
4)	Alk-Paper + Bacteria*	36.1	46.5	44.0	45.1	42.9	22.6
5)	U.V. Alk-Paper + Bacteria**	37.3	52.7	48.3	46.9	46.3	24.4
6)	Spartina	37.5	32.8	24.9	24.0	30.0	10.1

*Alkali treated filter paper pre-seeded with isolate JB-1
**Filter paper was U.V. irradiated for 48 hrs, alkali treated, pre-seeded with isolated JB-1, and then placed in the environment.

TABLE 10

Cellulose Fiber Solubilization Rates*

Study Area	Solubilization Rate
Inlet of the Baltic (low nutrient levels)	0.5
Unpolluted River Fyris	7.0
Unpolluted lake bottom (nutrient rich)	5.
Heavily polluted paper mill effluent	0.0
Sewage treatment plant settling tank	13.0

*Calculated from Hofsten and Edberg (1972).

in the present study for untreated cellulose fibers.

Chitin decomposition studies by Hood (1973), however, indicate much higher rates of almost 90 mg substrate solubilized/day/gm chitin for the Airplane Lake area. This high rate may indicate the importance of non-saccharide organic components, e.g., nitrogen, in the chitin polymer. Fookson and Frohnsdorff (1973) have speculated that nitrite may be photografted onto the cellulose chain during irradiation, thus accounting for increased degradability of treated cellulose (as opposed to acting as a catalyst or photosensitizer in the photolysis of cellulose).

In general, very little cellulolysis will occur in environments where growth conditions are not favorable for cellulolytic microorganisms. Subsequently, where a limiting growth factor might be available nitrogen, as in unpolluted, open seawater, cellulose solubilization may vary in direct proportion with nitrogen availability levels (Jewell, 1971). In very productive, nutrient-rich waters, however, cellulolysis may vary more directly with parameters such as the amorphous content of the cellulose fiber or surface area available for bacterial action. Degradation of cellulose in either nutrient-rich or-poor water appears to be accelerated by pre-seeding, thus, increasing possibilities of interaction of the cellulose fiber and decomposing bacteria.

Laboratory Cellulolysis Studies

In vitro degradation of various cellulosic substrates was compared for three cellulolytic isolates (Table 11). The results are of particular interest when noted with respect to the degree of polymerization values which were determined in a previous section. Avicel powder was solubilized at almost the same rate as Whatman CF-11, although the DP of the two celluloses are 478 and 672, respectively. The high degree of crystallinity in Avicel (microcrystalline cellulose) appears to make it more resistant to degradation than other less crystalline celluloses of similar DP, such as the ball-milled CF-11 (DP 432).

Table 11 shows that isolate JB-3 gave very low rates of decomposition. Similar results were shown for a Vibrio sp. by Kadota (1956). As previously discussed, JB-3 exhibited rapid maceration of cellulosics but produced little actual lytic activity towards fibers. Figures 14 and 15 show the attack of cellulose fibers by cellulolytic isolates JB-1, Cm, and JB-3. The former, JB-1, (top Fig. 14) and Cm (Fig. 15), may be seen to attack the fibers along the whole length of fiber. The entire fiber matrix disaggregated and is slowly solubilized (Figure 15, bottom). Fig. 14 (bottom) shows that JB-3 acts in more localized attack. The fiber is locally disaggregated but is not attacked along its whole length.

TABLE 11

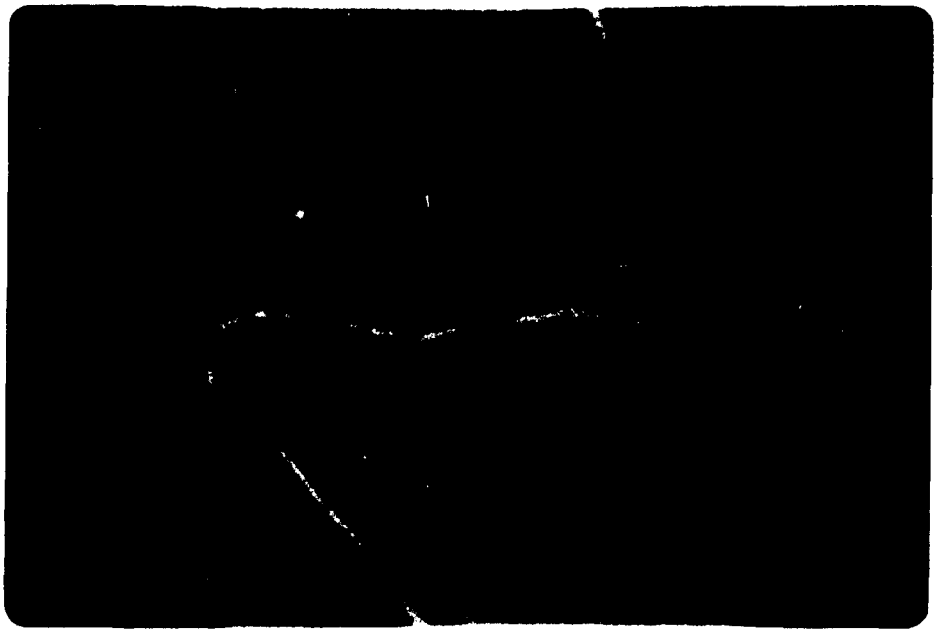
Residual Cellulose After Deterioration* for 5 Days by Cellulolytic Isolates. Results are given in percent.

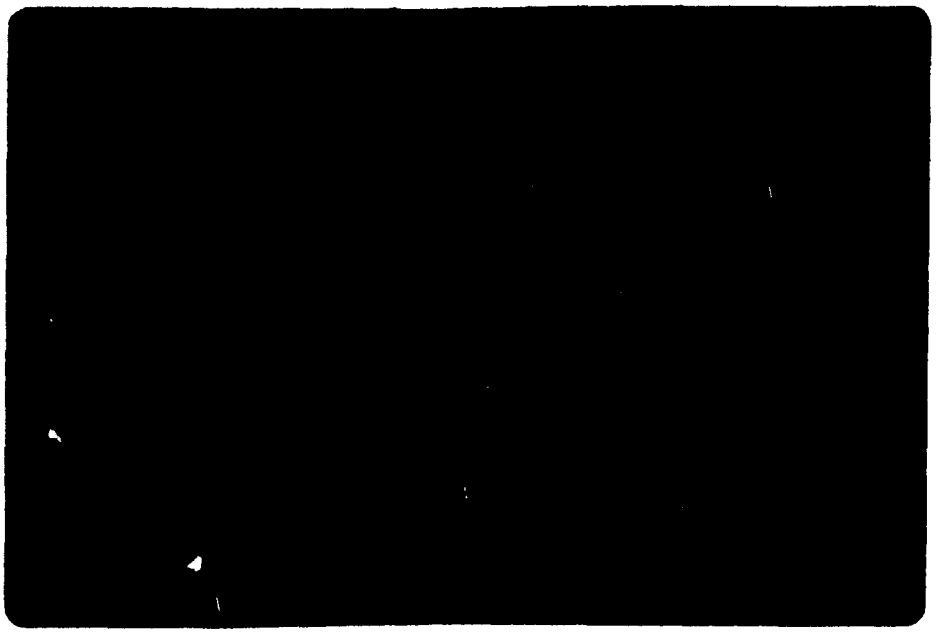
Substrate	Isolate JB-1	JB-3	Cm
Whatman CF-11	90.3	96.8	90.0
Ball-mill CF-11	84.0	91.8	82.9
Avicel PH-101	90.0	96.4	89.7
Brown Paper Towel	76.9	90.5	77.8
Filter Paper	92.5	98.1	85.9

*Degree of solubilization was determined gravimetrically after the organism was inoculated into 50 ml media containing each substrate as the sole carbon source. Incubation was for 5 days at 30 C on a reciprocal shaker.

Figure 14. Attack of cellulose fibers by cellulolytic isolates (JB-1, top; JB-3, bottom).

Figure 15. Attack of cellulose fibers by cellulolytic isolate Cm.





This results in maceration of the substrate with low total subsequent solubilization.

The effect of pretreatment on in vitro solubilization rates is shown in Table 12. U.V. irradiation produced some accelerating effect on decomposition, but not as great as that obtained with the alkali treatment. The combined U.V.-alkali process did not increase degradation rates considerably more than did the alkali treatment alone. A previous study had indicated that U.V. irradiation might have more of an effect on the degradation of paper towels because of its higher lignin content. In that study, Eskins et al. (1973) showed that lignin fractions of cellulose were made more susceptible to microbial utilization after U.V. irradiation. However, these results show that irradiation had little accelerating effect on degradation of paper towels.

Patterns of in vitro cellulolysis confirmed the results obtained from in situ determinations. Alkali swelling which presumably increases the bacterial- enzyme susceptability of the cellulose fiber, was shown to influence degradation rates more than did DP itself. Also, it was confirmed that the terrestrial isolate, Cm, maintained its cellulolytic activity (although lower than that indicated by Han, 1969) under conditions simulating a marine environment.

TABLE 12

Effect of Pretreatment on Solubilization of Cellulose Substrates by Isolate JB-1

Pretreatment	Substrate	% Solubilized*
1) None	Whatman CF-11	9.7
	Paper Towel	23.3
2) Alkali	Whatman CF-11	22.5
	Paper Towel	28.1
3) U.V.** + $NaNO_2$	Whatman CF-11	14.3
	Paper Towel	24.0
4) 2 & 3 Treatment Combined	Whatman CF-11	24.7
	Paper Towel	31.0

*Solubilization was determined as in the preceding Table.

**U.V. irradiation period was 48 hrs.

Polychlorinated Biphenyl Determinations

In the preceding sections, alkali pretreatment and U.V. irradiation were discussed as methods for accelerating the rate of biodegradation of cellulosic substrates. The effects of these treatments on cellulose were studied. However, other compounds, such as PCB's (Senum, 1973), are associated with cellulosics and also, presumably, may be affected by pre-treatment processes. Therefore, a series of experiments were performed to indicate the presence of PCB compounds in cellulosics and to show the effect that pretreatment might have on such compounds.

Prepared Samples

Cellulose powder, CF-11, was prepared to contain 5 ppm of PCB, Aroclor 1260. Chromatographic analysis of the powder shows 11 well defined peaks (Fig. 16). Alkali treatment of the prepared powder by autoclaving 10 minutes at 121 C with 1% sodium hydroxide had no effect on the PCB level as determined chromatographically (Fig. 17). The stability of PCB compounds to acid and alkali have been noted by other investigators (Edwards, 1971). A micro-scale alkali treatment for use in PCB residue confirmation and sample cleanup has been described by Young and Burke (1972). Their study showed that PCB's of 21-60 percent average chlorine content were completely stable to alkali

Figure 16. Gas chromatographic analysis of Aroclor 1260 standard.

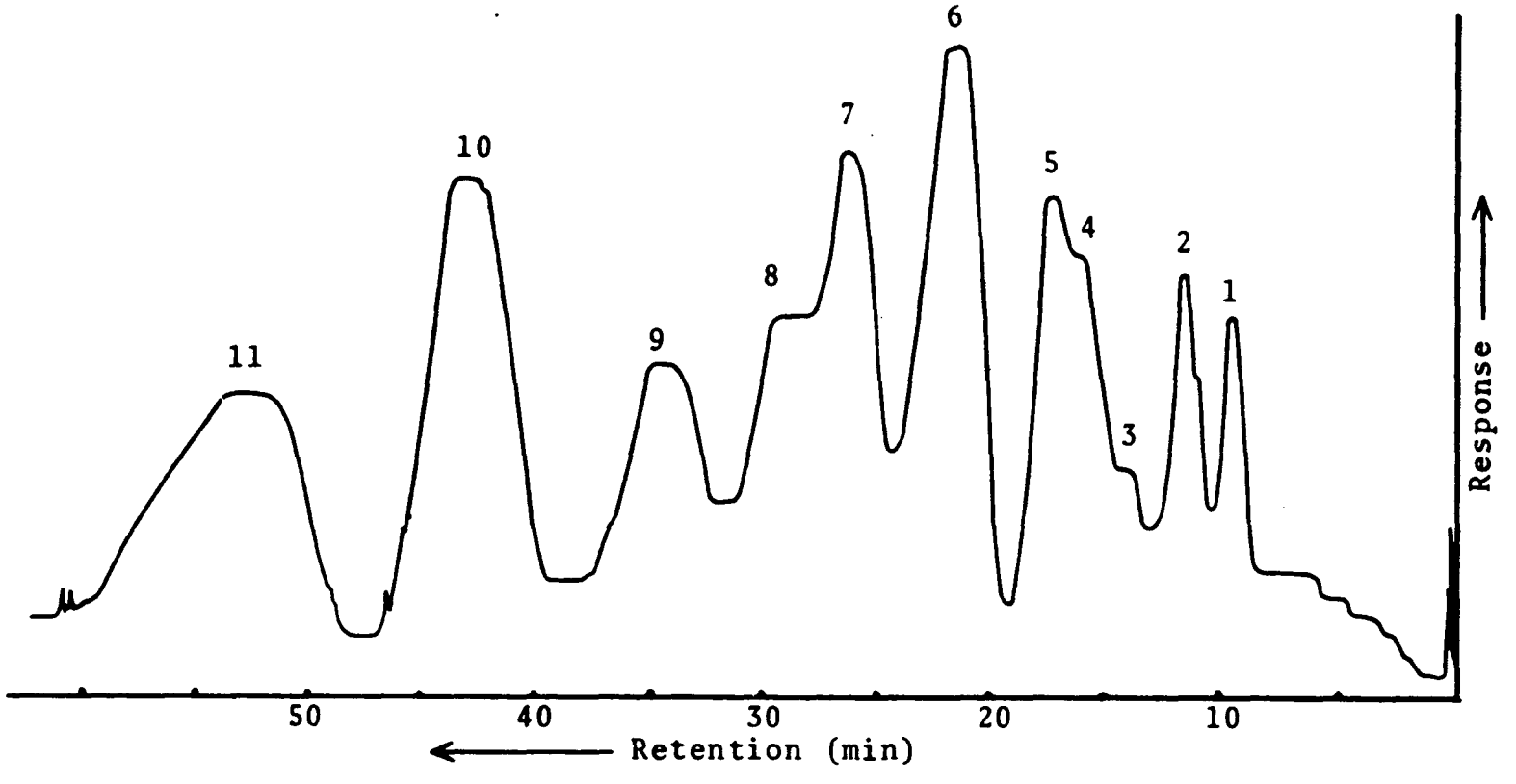

11
10
9
8
7
6
5
4
3
2
1
Response
50
40
30
20
10
Retention (min)

Figure 17. Gas chromatograph of alkali-heat treated Aroclor 1260.

while most of the chlorinated hydrocarbon pesticides were de-chlorinated or further decomposed. Alkali treatment also served to clean up extracts of samples containing non-volatile fatty substances as well as those with non-pesticidal substances which give rise to electron capture response, e.g., sulfur.

Consequently, alkali treatment of cellulosic materials may degrade certain pesticides such as DDT, Methoxychlor, and other organochlorinated compounds (Young and Burke, 1972). However, PCB's are resistant to the treatment, remaining stable and associated with the cellulosic substrate.

Hutzinger et al. (1972) and Safe and Hutzinger (1971) reported that PCB compounds were degraded during exposure to sunlight or U.V. The results shown in Table 13 confirm the former findings and indicate that after 48 hrs, only 6.2% of the original concentration of Aroclor 1260 remained. The effect of incorporating nitrite into the irradiation mixture, as in the previous cellulose pretreatment, is shown in Table 14. The data from the previous two tables are summarized pictorially in Fig. 18. A 50% reduction in PCB concentration was reached in about 6 hours without nitrite and in 4 hours with the additive. Preliminary experiments by Khan (1974) indicate even more rapid reduction rates of some of the less highly chlorinated Aroclors and possible utilization of other ions as photosensitizers.

TABLE 13

Effect of U.V. Irradiation on Aroclor 1260 Levels in Cellulose Powder

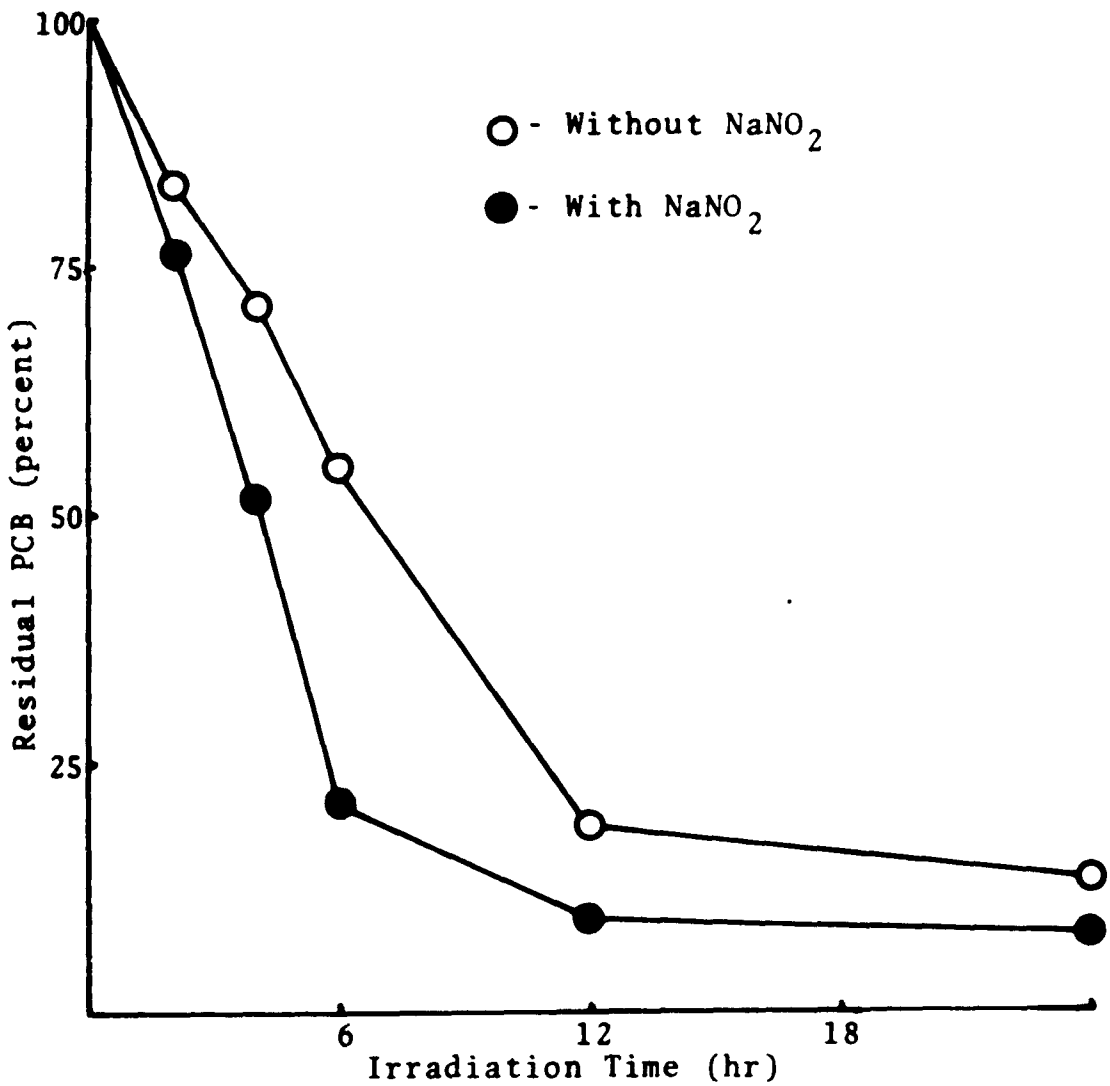
Irradiation Time (hrs)	Total Peak Height (Cm)	Percent Reduction	PPM
0	65.9	0	5
2	55.0	16.5	4.18
4	46.9	28.7	3.57
6	35.9	45.4	2.73
12	12.4	85.7	0.94
24	8.9	86.5	0.68
48	4.1	93.8	0.31

TABLE 14

Effect of U.V. Irradiation, with 0.1% $NaNO_2$, on Aroclor 1260 Levels in Cellulose Powder

Irradiation Time (hrs)	Total Peak Height (Cm)	Percent Reduction	PPM
0	56.0	0	4.30
2	42.7	23.7	3.28
4	29.1	48.1	2.23
6	11.8	79.0	0.90
12	5.4	90.4	0.41
24	4.4	92.1	0.34
48	1.3	97.8	0.10

Figure 18. Effect of nitrite presence on the U.V. photolysis of Aroclor 1260 in cellulose powder.


100
75
50
25
Residual PCB (percent)
○ - Without $NaNO_2$
● - With $NaNO_2$
6
12
18
Irradiation Time (hr)

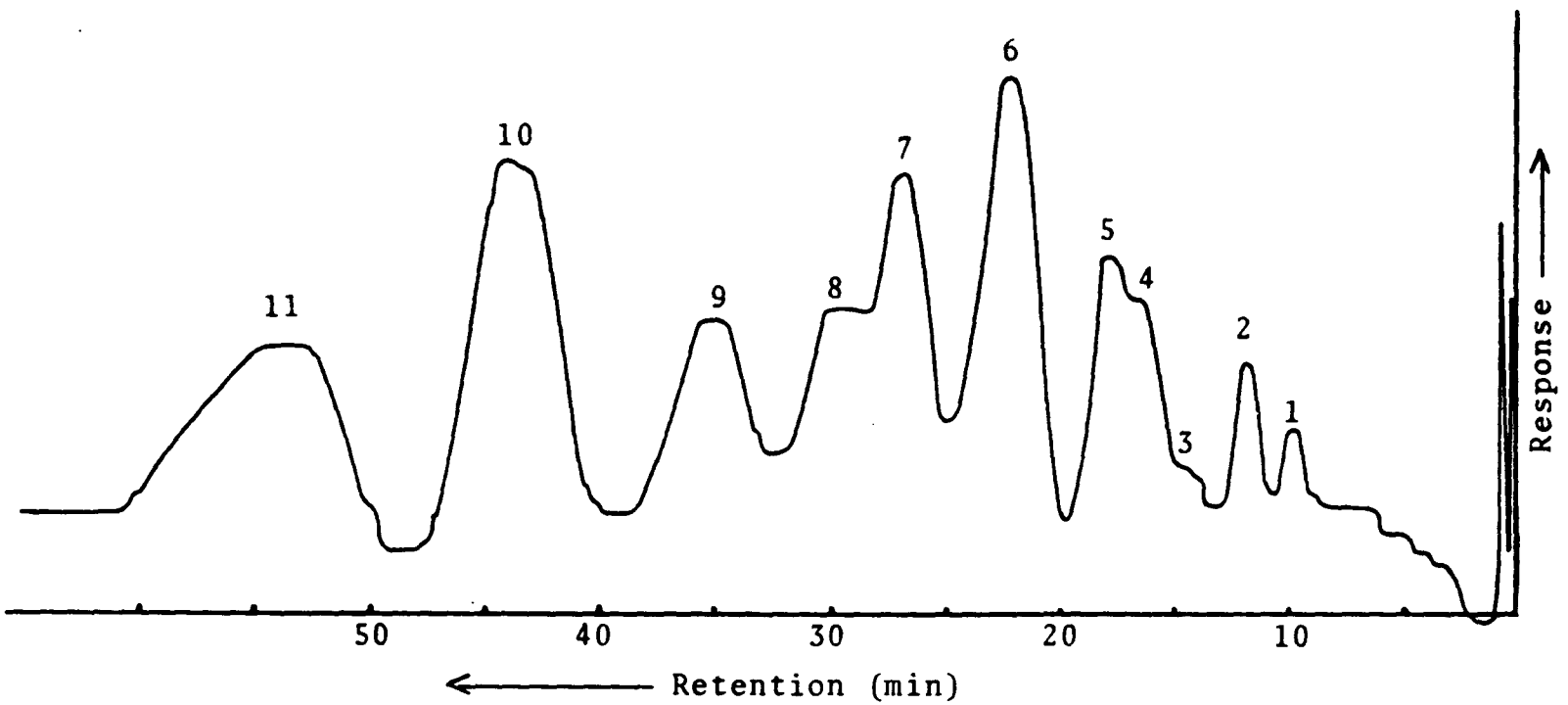
After 48 hrs irradiation, the sample with nitrite showed only a 4% greater total reduction than the sample without nitrite. However, a 90% reduction was achieved in about 12 hours with nitrite as opposed to almost 48 hours without. Thus, the highly increased rate of photolysis with nitrite present, appears to be of much greater importance than the total degradation level achieved.

A chromatographic analysis of cellulose powder after irradiation for 4 hrs, with no nitrite present, is shown in Fig. 19. All of the control Aroclor 1260 peaks (Fig. 16) are present, however, there is a 28.7% reduction in PCB concentration. After 4 hrs irradiation with nitrite present (Fig. 20) a 48.1% reduction is evidenced. Also, peaks 3 and 4 have almost totally disappeared and a new peak preceding peak 1 has formed (with a retention time of 5.9 min). Development of the new peak may indicate accumulation of a less chlorinated PCB isomer in a sequential decomposition of Aroclor.

Twelve hours irradiation totally eliminated peaks 3,4,8,9, and 11 (Fig. 21). Figure 22 indicates the extent of decomposition after 24 hrs irradiation with nitrite present. Only 7.9% of the original concentration Aroclor remains and only 4 of 11 previous peaks are present.

Lane (1973) reported 36% reduction in Mirex levels after U.V. irradiation for 48 hrs. There also was shown to be a linear relationship between Mirex remaining

Figure 19. Gas chromatograph of cellulose-PCB extract after 4 hrs U.V. irradiation with no nitrite present.


11
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9
8
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6
5
4
3
2
1
Response
50
40
30
20
10
Retention (min)

Figure 20. Chromatograph of 4 hr irradiated cellulose-PCB with 0.1% nitrite present.

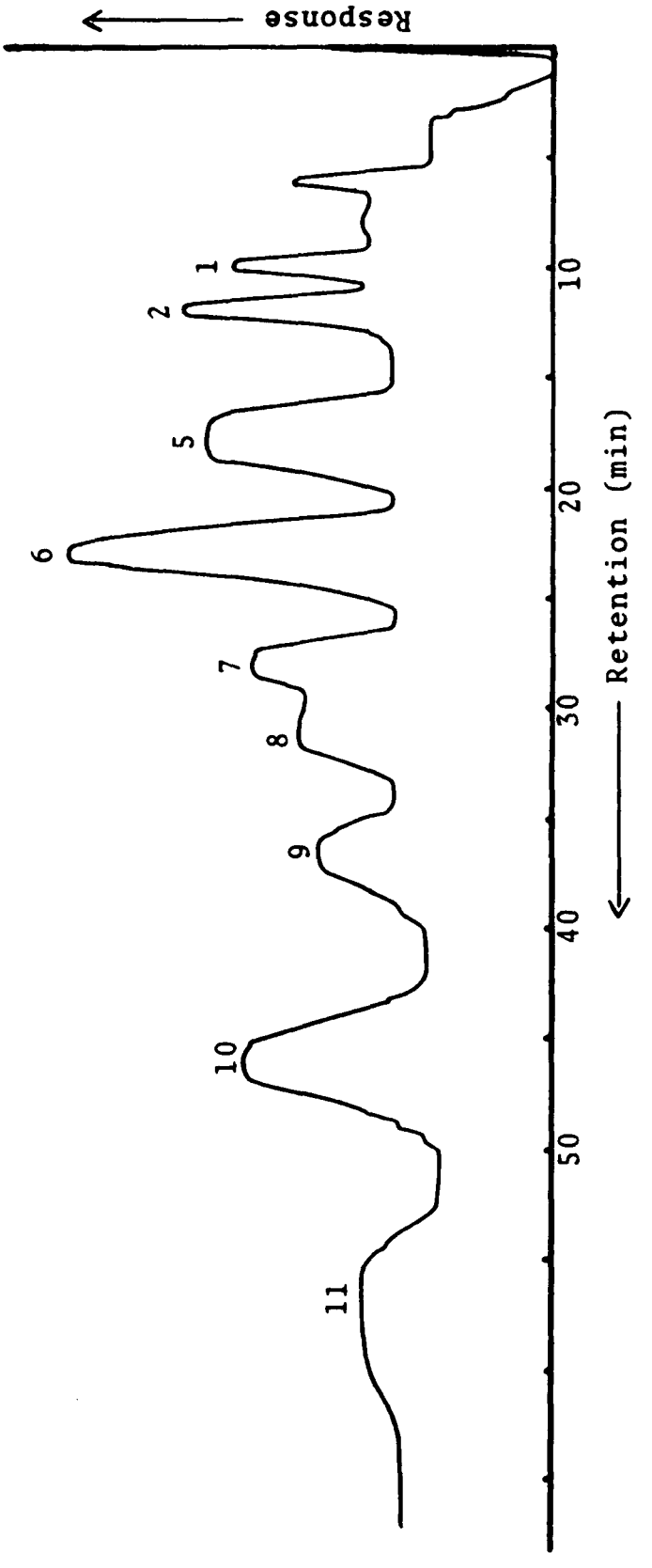

Response
1
2
5
6
7
8
9
10
11
10
20
30
40
50
Retention (min)

Figure 21. Chromatograph of 12 hr irradiated cellulose-PCB with nitrite present.

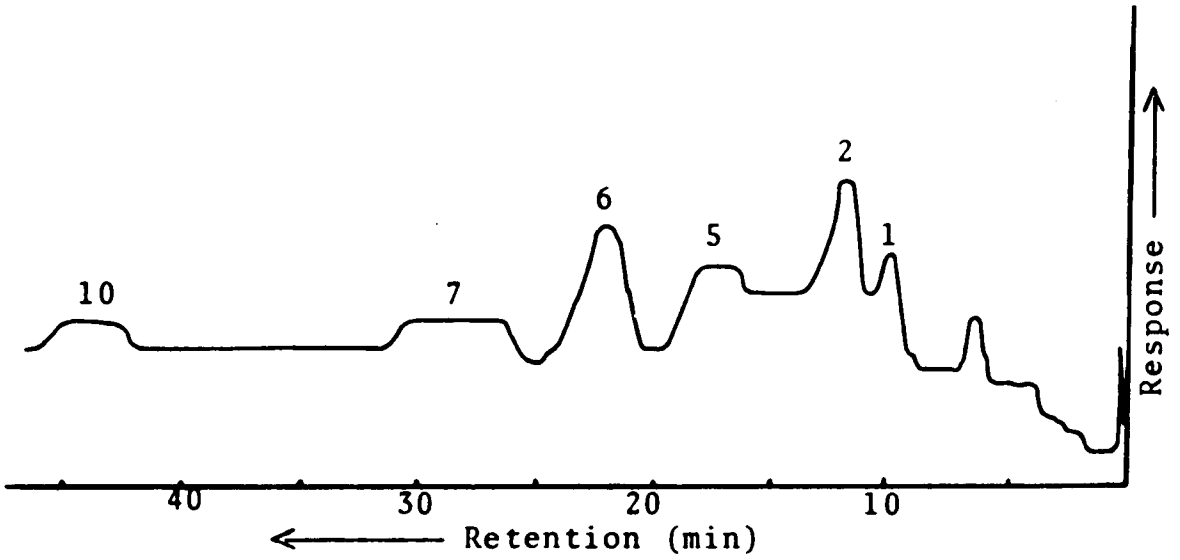
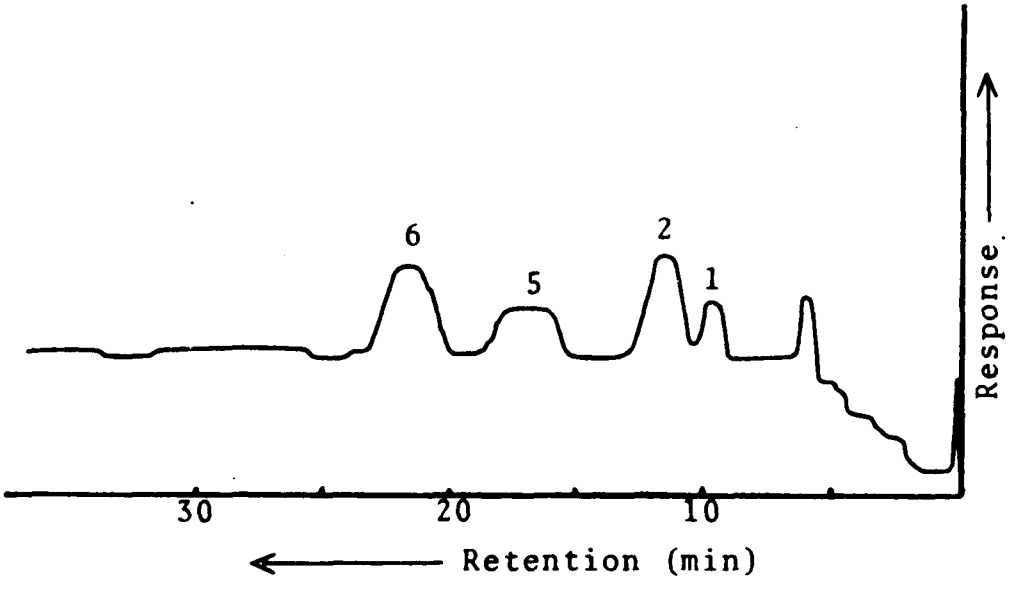

10
7
6
5
2
1
Response
40
30
20
10
Retention (min)

Figure 22. Gas chromatograph of 24 hr irradiated cellulose-PCB with nitrite present.


6
5
2
1
Response
30
20
10
Retention (min)

and length of time of U.V. exposure. A linear rate of decomposition may occur in some PCB isomers, however, Fig. 18 shows a non-linear rate of degradation for the PCB compound Aroclor 1260. The decomposition pattern may be the result of varying sensitivity to photolysis of the individual isomers (over 240 possible isomers, Kinoshita and Sunada, 1972) which are constituants of the above compound.

Substrate Irradiation Proposal

Recent studies by Harvey et al. (1973) have indicated that not only are the occurrence and levels of PCB's increasing in the environment, but also that their environmental half-life appears to be considerably greater than that of DDT and its metabolites. Additionally, the latter compounds have been implicated as an unrecognized source of PCB's. Maugh (1973) has proposed a mechanism for sunlight degradation of DDT whereby PCB's may be enriched in the less highly chlorinated compounds. Thus, concern and research seem well warranted in lowering levels of contaminating materials which will enter the human food chain.

The above results indicate that PCB levels in cellulose may be reduced by 90+% by appropriate irradiation treatment. Kinoshita and Sunada (1972) have described an ionizing radiation (Co-60) treatment for reduction of low levels of PCB contamination in waste water supplies. They indicate that the process is both economically

(8 cents/1000 gal) and technically feasible. Perhaps further research is justified to develop a similar process for the reduction of PCB's in cellulosic wastes used in various recycling and cellulose utilizing operations, i.e., production of SCP from microbially degraded cellulose.

Upon examination of the LSU-SCP pretreatment process (Appendix E), it appears that an irradiation treatment could be incorporated with little alteration to the overall operation. Stock-piled cellulosic material could be irradiated during storage, prior to alkali pretreatment. Or, the ground substrate could be irradiated in agitated slurry tanks either before or after alkali addition. Perhaps the existing slurry tanks, T_1 and T_4 (Fig. 2), could be modified to allow irradiation during alkali slurrying and again after heat processing (Oxidation Oven). In addition to the cellulose photolysing and PCB degrading activity of U.V. irradiation, further benefit might be realized in that the load of contaminating microorganisms entering the microbial treatment section of the process would be lessened with consequent alleviation of contaminant fouling and viral problems in SCP production.

Cellulosic Waste Analysis

The presence of PCB's in a common cellulose product was determined by methods similar to those described by Dube et al. (1974). Brown paper towels were extracted and analyzed by gas chromatography. A chromatographic analysis

is presented in Fig. 23. This chromatograph was compared with that of various PCB standards and found to be similar to that or Aroclor 1254 (Fig. 24). Peaks from towel extracts corresponded to 6 of 8 major electron-capture peaks in the standard chromatograph. Table 15 presents the peak retention times, their heights, and amount of PCB represented by the chromatograph. A concentration of 4.6 ppm was determined for the paper towel sample. This value is only approximate because of the relative method of comparing standard and unknown chromatographs. However, the value does fall within a 2 to 40 ppm (as equivalent PCB's) range of PCB concentration determined by Senum et al. (1973) in a survey of 100 papers and paper products. Their study also reported the same number of electron-capture peaks (16 peaks) for samples of brown paper towel.

Further experiments with brown paper towels were performed to determine the fate of associated PCB compounds during microbial cellulolysis. When uninoculated paper towel, in a basal salts solution, was incubated at 30 C on a rotary shaker, PCB residues were found to be distributed in both the solid and liquid phases of the mixture (Table 16). Although PCB's are quite insoluble in water, what may be described as a colloidal aqueous solution can be obtained. Kinoshita and Sunada (1972) achieved "solutions" of 100 ppb concentration of PCB

Figure 23. Gas chromatographic analysis of brown paper towel extract.

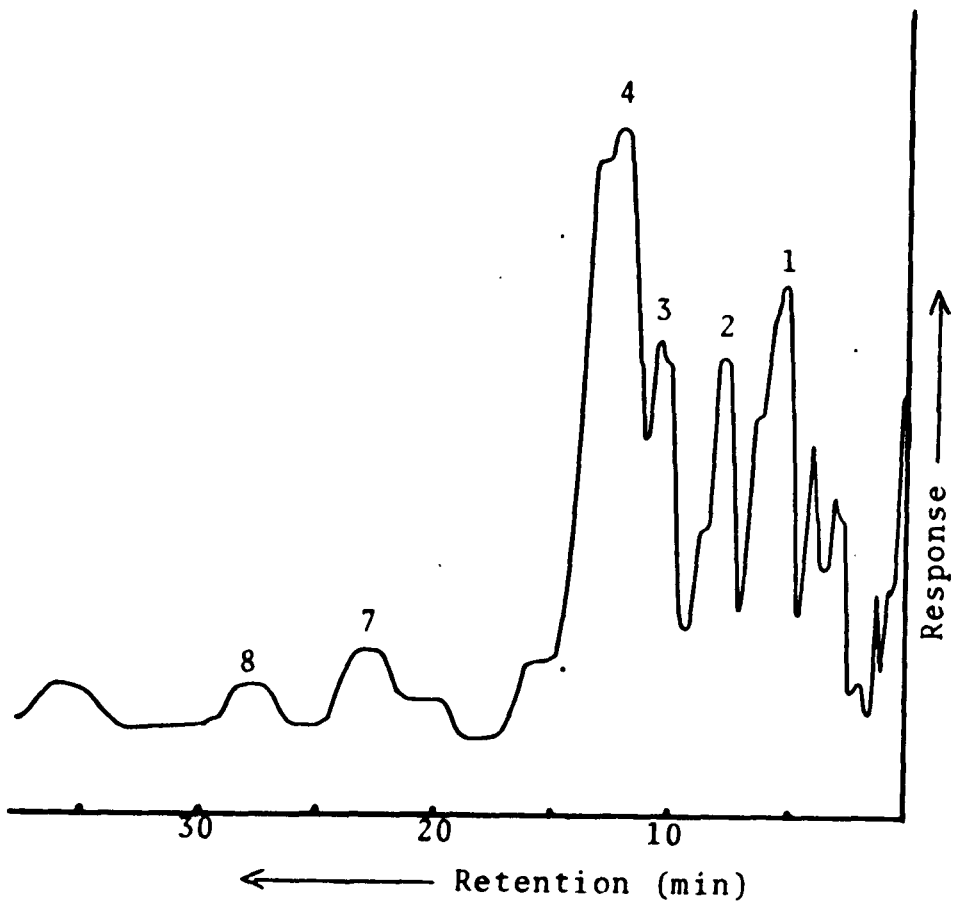

4
3
2
1
8
7
Response
30
20
10
Retention (min)

Figure 24. Gas chromatographic analysis of Aroclor 1254 standard.

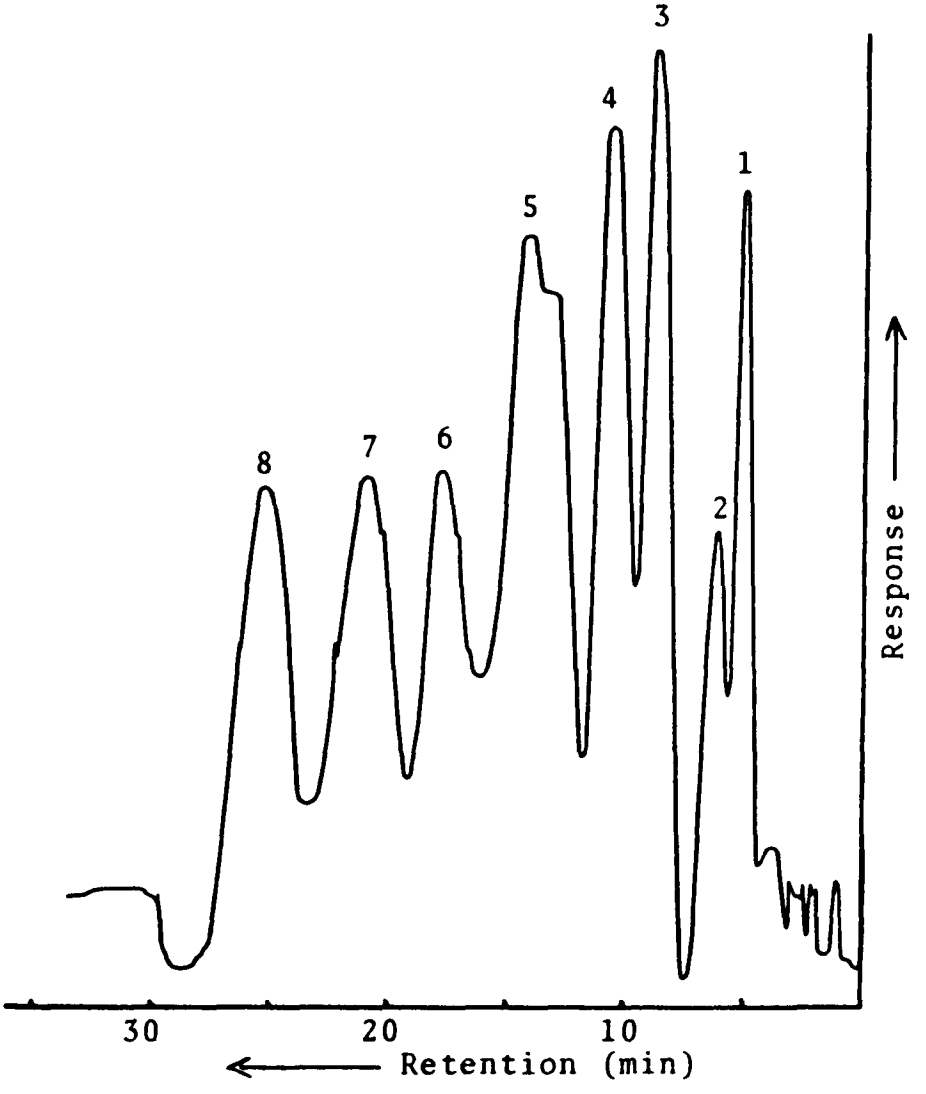

3
4
1
5
8
7
6
2
Response
30
20
10
Retention (min)

TABLE 15

Electron-capture Response Correlation
for Aroclor 1254 Standard
and Brown Paper Towel Extract

	Retention time (min)		Peak height of Corresponding peaks (Cm)	
Peak No.	Standard	Towel	Standard	Towel
1	5.0	5.0	14.7	6.9
2	6.0	6.1	8.5	5.5
3	8.5	8.6	18.0	6.3
4	10.5	10.5	14.5	11.0
5	14.0			
6	17.3			
7	20.7	20.8	6.4	1.9
8	24.3	24.1	7.6	0.9
		Total Peak Height	69.7	32.5
		PPM	10.0	4.6*

*Total towel peaks equal 46.6% of the total peak height obtained from corresponding peaks of a 10 ppm standard. Thus, the towel peak total represents an approximate PCB level of 4.6 ppm.

by shaking liquid suspensions for half an hour. Table 16 shows 3.1 ppm in the pelleted material and 0.8 ppm in the liquid phase.

When the above experiment was repeated with the cellulolytic isolate (Cm) added, a different pattern of PCB partitioning resulted. All of the recoverable PCB was present in the solid phase of the culture mixture. It may be noted that in both the irradiation and paper towel experiments, PCB recovery rates for the liquid extractions were not as high as for dry extraction. Approximately 85% extraction efficiency was obtained by the liquid extraction used in the paper towel experiments (Table 16).

Gas chromatographic analyses of uninoculated and inoculated towel (pellet) extracts are shown in Figures 25 and 26 respectively. PCB residues are shown to be concentrated in the microbial-cellulose mass. Approximately 18% of the total PCB concentration, which has previously been dispersed in the liquid phase, was concentrated into the solid phase resulting in a 22.5% increase in PCB level for the solid phase (Table 16).

Dube et al. (1974) reported similar findings for the removal of PCB residues from wastewater digestion treatments. He reported approximately 70% removal of the residues with subsequent concentration of PCB's in treatment digester sludges. Numerous other studies (Sodergren, 1972; Wildlish and Zitko, 1971, etc.) have

TABLE 16

Effect of Bacterial Cellulolysis
of Brown Paper Towels
on Equivalent PCB Concentration

Sample		Total Peak Height (Cm)	Percent of Control	PPM
Dry Towel Control		32.5		4.6
Towel + Basal Salts Uninoculated	Pellet*	21.6	66.5	3.1
	Supernatant	6.0	18.4	0.8
Towel + Basal Salts + Isolate Cm.	Pellet	28.2	86.8	4.0
	Supernatant	0	0	0

*After incubation for 5 days at 30 C, residual fiber and bacterial cells were harvested by centrifugation. The initial supernatant and pellet were retained for analysis; subsequent washings of the pellet were discarded.

Figure 25. Gas chromatographic analysis of pellet extract from uninoculated brown paper towel-basal salts solution mixture.

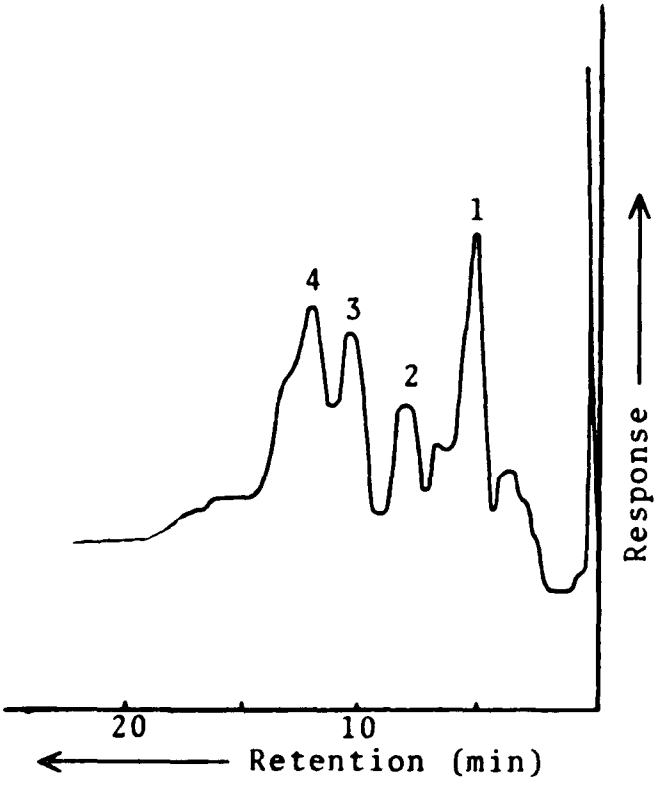
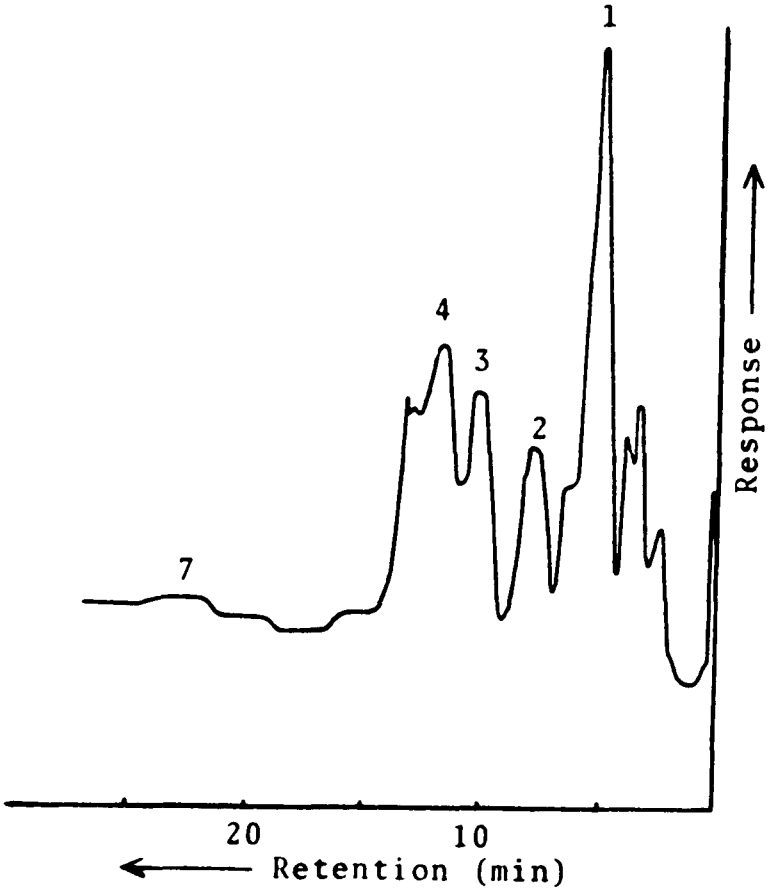

1
4
3
2
Response
20
10
Retention (min)

Figure 26. Gas chromatographic analysis of pellet extract from inoculated brown paper towel-basal salts solution culture.


1
4
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2
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Response
20
10
Retention (min)

shown that PCB's may be concentrated by microorganisms and passed through the food chain undegraded.

These findings indicate that PCB compounds are present in commercial cellulose products at levels approximating the maximum limits (5 ppm) recommended by the FDA as safe for human consumption. If cellulosic wastes are microbially degraded to produce single cell protein, PCB's are concentrated in the cellulose-bacteria matrix and incorporated into the end product of the process. U.V. irradiation may be used to lower PCB levels in cellulosic substrates by as much as 90%. Further study may be warranted to determine the exact nature of PCB incorporation into the bacterial biomass. It is assumed (Edwards, 1971) that the compound is accumulated in cellular lipid fractions. However, adsorption phenomena may play an important role in uptake prior to incorporation into lipid material.

Additional experiments are being performed with isolate Cm, utilizing a PCB contaminated, soluble cellulose substrate (CMC). The solubility of the substrate will facilitate cell crop harvesting and washing to remove any residual substrate. Thus, an actual PCB uptake may be determined in the Cellulomonas sp.

SUMMARY AND CONCLUSIONS

Cellulose decomposition processes are a function of a multitude of environmental and substrate mediated factors. In this study, an attempt has been made to evaluate some of these factors and to examine various considerations in the expanding technology of cellulose waste utilization.

The concept of cellulose utilization for the production of food or industrial commodities is not new. A number of food yeast production schemes, involving acid hydrolysis of cellulose, were developed during World War II. However, in view of current developments in both levels of world food resources and in technological capabilities, there is sufficient basis for re-examination of various food generating processes which previously have been regarded as infeasible. Vast amounts of cellulose are present in food and agricultural waste streams and represent an invaluable replenishable resource. Conversion or recycling of cellulose through accelerated biotransformation processes is one approach in the efficient utilization of cellulosics. Thus, there is a necessity for understanding the mechanisms of cellulolytic microbial action, cellulose degradation kinetics, and the impact of potentially toxic compounds occurring concomitantly with cellulose.

Initially, a double-layer agar system was developed for the isolation of cellulolytic bacteria to be used

in later degradation studies. Alkali pre-conditioning of ball-milled cellulose, for incorporation into an agar overlay, increased the rate of clearing zone formation as much as 4 times the rate for clearing in conventional (Gylswyk, 1970; Hazeu and Eggins, 1966) cellulose agar preparations. Diagnostic cleared zones were formed in the modified cellulose-agar overlay in 5 days at 30 C. The system was shown to have limited application in the enumeration of cellulolytic bacterial populations. Low colony density must be maintained on plates and it must be recognized that numerous colonies of cellulolytic species, e.g., Vibrio sp., may develop with no clearing of the cellulose agar. Studies are being continued to improve the isolation/enumeration technique. Incorporation of an indicator dye may prove useful in the enumeration of cellulolytics which cause no clearing zones in the cellulose-agar overlay.

Pretreated cellulose samples were solubilized in cadoxen and analyzed for determination of their degree of polymerization (DP). Ball-milling or UV-nitrite irradiation lowered the DP to a greater extent than did alkali treatment or UV-no nitrite irradiation. DP values were examined with respect to bacterial degradation rates for various cellulose samples to determine if DP reduction was concurrent with accelerated cellulose decomposition. The results indicated that in effecting an increase in cellulolytic rates, DP is of secondary importance compared

to the degree of swelling or bacterial accessibility of the cellulose fiber. DP reduction is an integral part of cellulose decomposition, however, low DP, highly crystalline substrates were shown to have lower digestion rates than did higher DP, less crystalline celluloses.

Laboratory and in situ studies were used to determine if appropriate pretreatment of cellulosic substrates could decrease their recycling time in the environment. A combination of nitrite-photochemical, alkali, and bacteria seeding pretreatments was shown to increase the rate of cellulose solubilization by a factor of 6.5. A low rate of 3.8 mg solubilized/24 hrs/gram substrate was determined for the decomposition of untreated, purified cellulose (filter paper). The introduced (filter paper) substrated was degraded, in situ, at a rate 2.7 times slower than that for the indigenous substrate, Spartina. This indicates that processed cellulose wastes are even more recalcitrant than are natural cellulosics and may, therefore, be particularly susceptible to sequestered elimination from recycling processes.

Further experimentation should include determination of cellulolytic activity levels in a deep ocean environment. Preliminary studies by Jannasch and Wirsen (1973) indicate that although there are high populations of viable, heterotrophic microorganisms in deep sea sediment, metabolic activity is extremely low. The interacting effects of

pressure and temperature appear to cause fundamental differences in the role played by microorganisms in the turnover of organic matter in the deep sea. However, the exact nature of these differences has not yet been fully elucidated.

Jannasch et al. (1971) have noted that rates of biodegradation in the marine environment have great implications with regard to the dumping of organic waste. Sieburth and Dietz (1974) and others (Jannasch and Wirsen, 1973) have reported data indicating the low biodegradability of cellulosic waste when dumped in the form of compacted blocks, intact containers, etc. Without proper pretreatment, such material may be effectively lost from transformation processes. Autochthonous or indigenous cellulose in the estuarine environment appears to be maintained in a steady-state flux. However, allochthonous materials may upset the homeostatic condition and cause a deterioration of the entire system.

In addition to the refractory nature of processed cellulosic materials, a compounding problem exists with respect to toxic contaminants associated with cellulose wastes. Research in developing the technology of increased cellulose utilization (Rogers, 1974; and Appendix C) must recognize and deal with the hazards of concomitant compounds.

Electron-capture gas chromatographic studies indicated

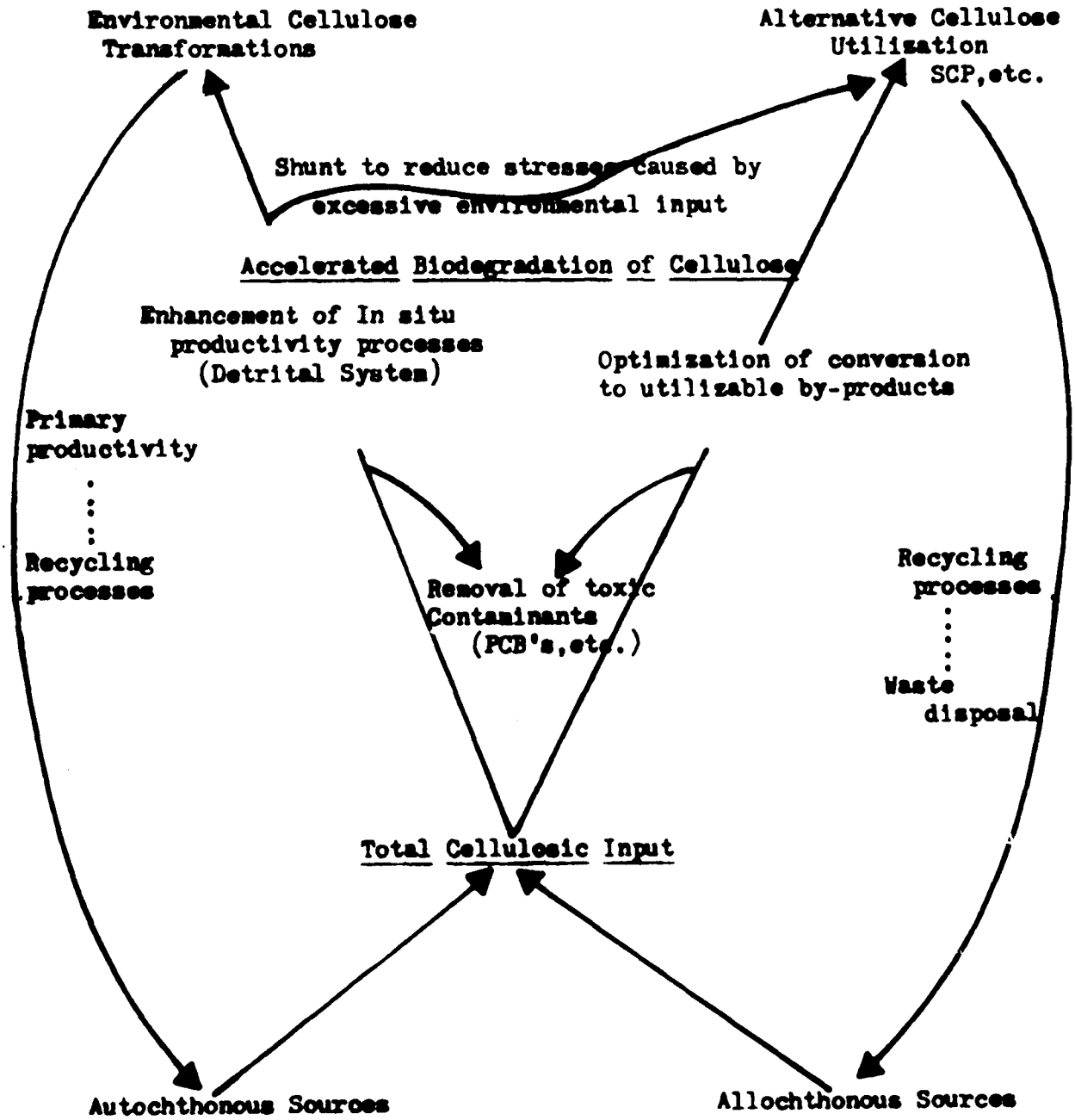
that a 90+% reduction of PCB residues could be effected by appropriate UV irradiation. PCB's in untreated cellulosic substrates were shown to remain stable throughout conventional cellulose decomposition processes and to be concentrated in the microbial-cellulose biomass during biodegradation. A proposal was presented which suggested incorporation of UV irradiation treatment into waste cellulose-SCP systems (or any recycling processes) where bioconcentration of PCB residues may present a significant potential danger.

Figure 27 is a schematic representing the interrelationships between cellulose transformation processes in the estuarine food-web cycle and in proposed cellulose utilization processes. It is readily apparent that man may lie at the apex of either cellulose recycling system. To better understand both the potential and limitations of cellulose processes it is requisite to determine in situ rates of biodegradation and the factors which mediate these rates. This study demonstrated that rates of cellulose decomposition, in situ, could be significantly increased by chemical/physical/microbial pretreatment of the substrate. Also, it was shown that any detrimental impact of such material on the environment could be lessened by pretreatment to lower levels of associated PCB residues.

The above environmental considerations parallel

Figure 27. Schematic of interrelationships in cellulose transformation processes.

CELLULOSE BIODEGRADATION PROCESSES



similar problems in microbial cellulose-SCP processes. Objectives of such increased cellulose utilization technology include maximizing substrate conversion to desirable compounds concurrent with removal of toxic materials. Both biotic and abiotic reactions must be stimulated to efficiently integrate cellulose wastes into the mainstream of our natural resource utilization. Development of this technology has the potential to make great strides toward alleviating both world protein shortages and problems being encountered in the disposal of solid wastes.

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APPENDICES

APPENDIX A

Cost Comparison of Proteins Obtained from Various Sources*

Protein Source	Percent Protein	Cost of Product $/ton	Cost of Protein $/ton
LSU-SCP	56	258	460
Protein from paraffins	50	140-170	280-340
Eggs	11	520	4,650
Chicken	14.5	550	3,640
Beef	11.5	393	3,420
Dried haricot beans	22	172	780
Wheat flour	11.7	80	680
Skimmed milk powder	36	185	510
Fish meal	80	400	500
Defatted soybean flour	51	200	400

*From Tyagi, 1972.

APPENDIX B

AMINO ACID PROFILE OF VARIOUS PROTEIN SOURCES

Values are given in grams of amino acid per 100 grams protein.

Amino Acid	LSU SCP	FAO Reference	Wheat Flour	Beef	B.P Protein
Arginine	9.21		4.2	7.7	5.1
Histidine	2.30		2.2	3.3	5.1
Isoleucine	4.74	4.2	4.2	6.0	4.6
Leucine	11.20	4.8	7.0	8.0	3.1
Lysine	6.84	4.2	1.9	10.0	6.0
Methionine	1.86	2.2	1.5	3.2	1.1
Phenylalanine	4.36	2.8	5.5	5.0	8.1
Tyrosine	2.67	2.8			
Threonine	5.37	2.8	2.7	5.0	11.0
Valine	10.71	4.2	4.1	5.5	7.0

From Srinivasan and Han, 1969.

APPENDIX C

SAMPLE CALCULATION FOR DEGREE OF POLYMERIZATION DETERMINATION

Polymer Concentration (C) g/100 ml	Flow (t) sec	Specific Viscosity* (n_{sp})	n_{sp}/C
0.3000	300.0	1.1428	3.8093
0.1500	210.4	0.5025	3.3500
0.0750	172.3	0.2310	3.0800
0.0375	155.4	0.1102	2.9400
0.0187	147.5	0.0536	2.8700

*Specific viscosity, $n_{sp} = (\frac{t}{t_o}) - 1$ (1)

t_o for solvent (cadoxen) = 140 sec.

A plot of n_{sp}/C versus C yields a limiting viscosity number, $[n]$, of 2.8 where C = 0.

Then, utilizing equation (2) - see Methods Section:

$$[n] = 2.85 \times 10^{-4} (\overline{M}w)^{0.76} \quad (2)$$

$$\overline{M}w = (2597.65\ [n])^{1.3158} \quad (3)$$

$$= (2597.65\ [2.8])^{1.3158}$$

Average Molecular Weight, $\overline{M}w$ = 120,580

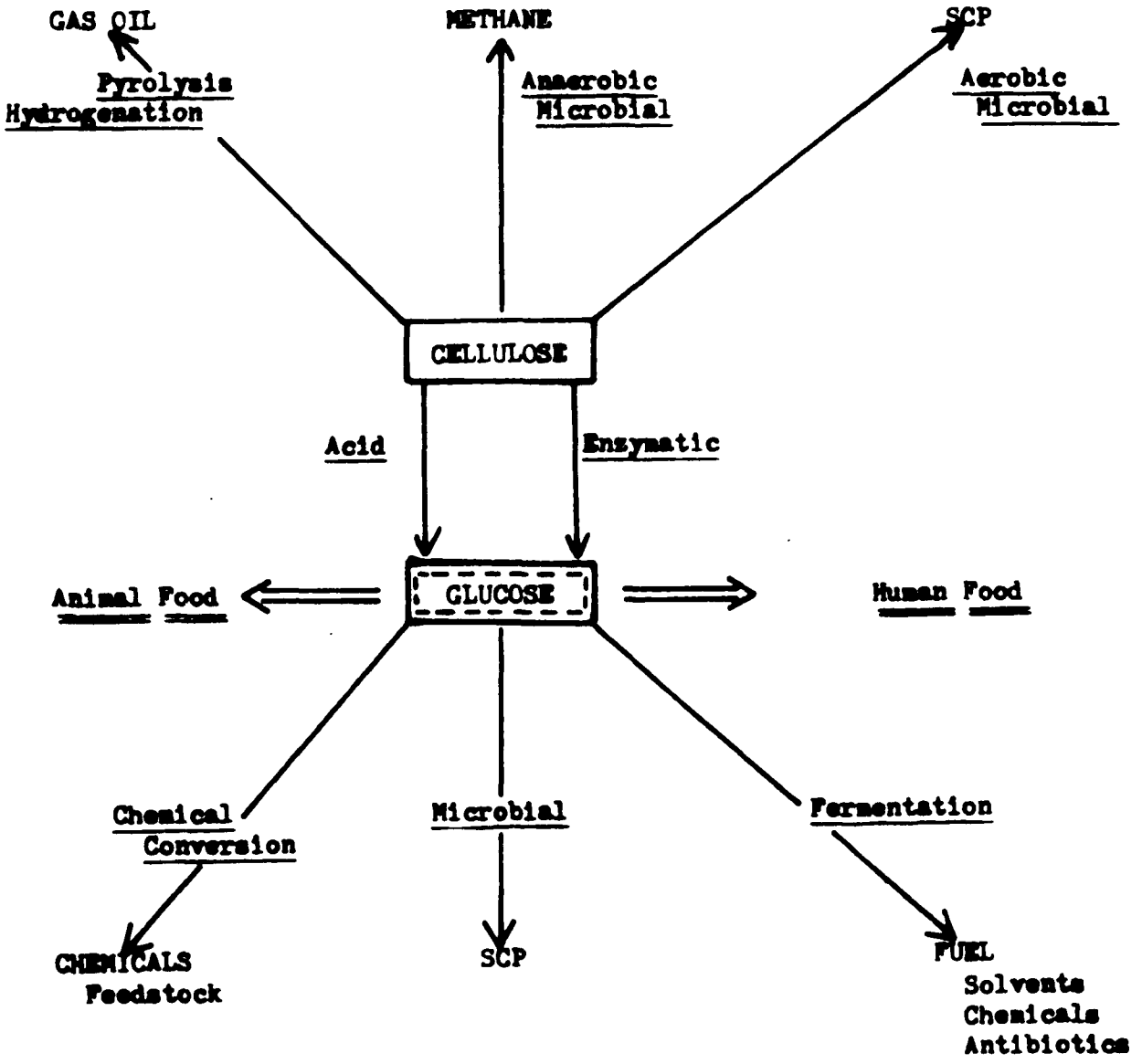
$$\text{Since DP} = \frac{\text{Molecular Weight of Polymer}}{\text{Molecular Weight of Polymer Unit}}$$

DP = 120,580/162

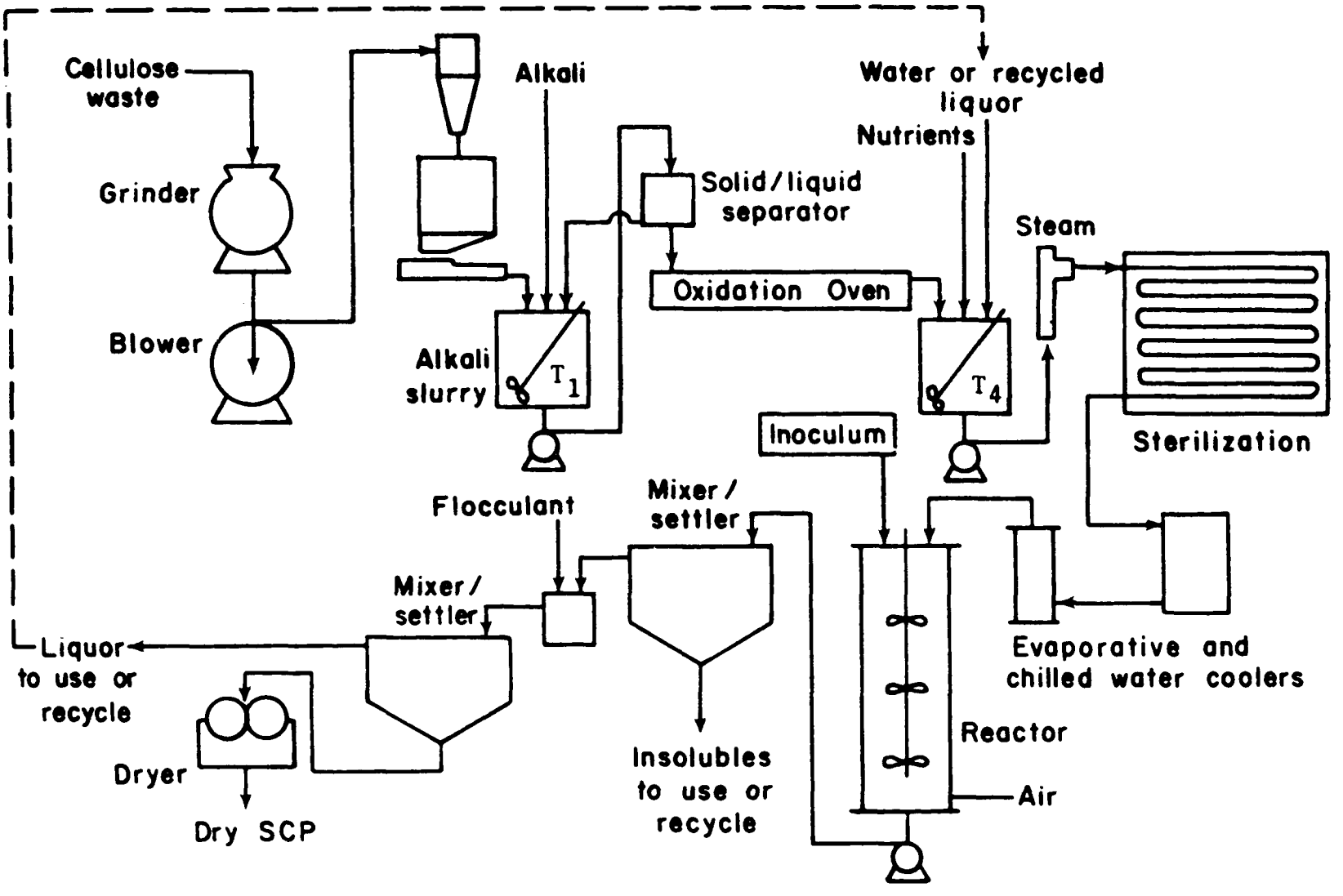
Therefore, DP = <u>744</u>

APPENDIX D

CELLULOSE UTILIZATION



(Adapted from: Mandels, 1974)



SCHEMATIC OF ALKALI TREATMENT OF CELLULOSE FOR THE LSU-SCP PROCESS. (Adapted from Srinivasan and Meyers, 1974.)

VITA

Herbert John Bavor, Jr., eldest son of Dr. and Mrs. Herbert J. Bavor, was born on November 15, 1946, in Cleveland, Ohio. He was graduated from Douglas MacArthur High School in Decatur, Illinois, in June 1964. In September 1964 he entered the University of Illinois at Urbana, and in June 1968 received the Bachelor of Science degree in Dairy Technology.

The following fall, he entered the graduate college at the University of Illinois in Urbana, where in August 1971 he received his Master of Science degree from the Department of Food Science.

He entered Louisiana State University Graduate School in January 1972, and is at present a candidate for the Doctor of Philosophy degree in the Department of Food Science.

EXAMINATION AND THESIS REPORT

Candidate: Herbert John Bavor, Jr.

Major Field: Food Science

Title of Thesis: Accelerated Biodegradation of Cellulosic Substrates

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

November 14, 1974